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THE ANTIDIURETIC ACTION OF YOHIMBINE

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IN A PREVIOUS COMMUNICATION (Fugo and Gross, 1942) it was shown that the alkaloid yohimbine has, as part of its systemic action, the ability to release excessive amounts of the luteinizing principle from the anterior lobe of the pituitary. It was of interest, therefore, to determine if the stimulating action of this alkaloid was confined to the anterior pituitary or whether it could also be demonstrated in connection with the posterior lobe.

Shen (1937) presented some evidence that yohimbine has an action on the melanosomotropic function of the pituitary when he demonstrated that a number of drugs including yohimbine are capable of producing a marked dispersion of melanosomes in normal frogs but that these same drugs had no effect on the melanosomes of hypophysectomized animals.

Zunz and Vesselovsky (1939) showed that both yohimbine and its isomere corynanthine when administered to normal dogs after ingestion of water to produce an aqueous diuresis, were capable of reducing the urinary output of these animals to a marked degree. These authors made no attempt to explain the mechanism of this antidiuretic effect.

It was the purpose of the present investigation to try to demonstrate that the antidiuretic effect obtained with yohimbine is due to a stimulation of the posterior pituitary causing it to release larger amounts of its antidiuretic hormone.

MATERIALS AND METHODS

These experiments were performed on female dogs of mixed breeds weighing from 6 to 16 kg. Three types of experimental animals were used. The first group was composed of 7 normal dogs. The second group consisted of 4 dogs with diabetes insipidus brought about by surgical interruption of the

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hypothalamic stalk. The last group of 2 were normal animals which were later completely hypophysectomized.

All animals were maintained on a standard diet of commercial dog biscuit (30 gm./kg./day). In some experiments the water intake was controlled by administration of definite amounts by stomach tube while in other cases the animal was given free access to water at all times. At the end of each 24 hour period the animals were catheterized, the total volume, specific gravity and, in some cases, urinary chlorides of the 24 hour urine sample were determined. In those experiments in which the animal was given free access to water the intake was recorded. The dogs were confined to metabolism cages continuously during all experimental periods.

Yohimbine was administered subcutaneously in doses of 2 mg./kg. from 1 to 3 times daily over a period of from 1 to 3 days depending upon the experiment.

RESULTS

Normal dogs. These animals were maintained on a constant diet and given water by stomach tube twice daily in the amount of 25 cc./kg. of body weight.

When the daily urinary output and specific gravity reached a

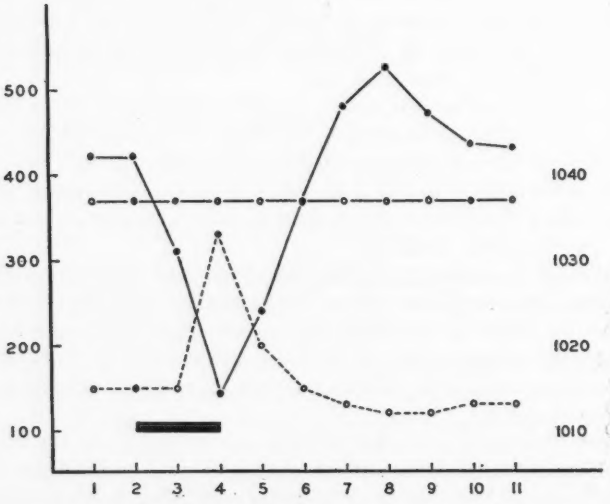

FIG. 1. Dog #2. Normal wgt. 7.3 kg.

●———● Urine output; ○———○ Water intake by stomach tube; ○- - - - - ○ Specific gravity; ▬▬ Period of administration of 2 mg. kg. of yohimbine HCl subcutaneously 3 times daily. Note the marked antidiuresis.

comparatively constant level yohimbine was injected subcutaneously in a dosage of 2 mg./kg. from 1 to 3 times during a 12 hour period for either 1 or 2 days in the various experiments.

During the period of drug administration and for the day following, the 24 hour volume of urine dropped markedly, and the specific gravity increased. After the antidiuretic effect had worn off the urine volume exceeded the pre-injection level and then returned to normal (fig. 1). When the drug was administered for one day only the maximum antidiuretic effect was observed the following day.

During the period of drug injection the animals exhibited slight signs of nervousness usually accompanied by mydriasis and some

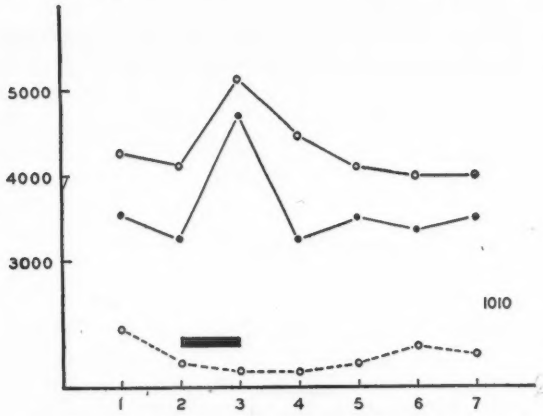

FIG. 2. Dog #4. With diabetes insipidus; wgt. 11.9 kg.
●——● Urine output; ○——○ Water intake; ○- - -○ Specific gravity; ▬▬ Period of administration of 2 mg. kg. of yohimbine HCl subcutaneously 3 times daily. Animal was allowed free access to water. Note the increase in urinary output and increase in water intake.

slight diarrhea. However these symptoms were always absent during the period of maximum antidiuretic effect.

Dogs with diabetes insipidus. In view of the observation made in the experiments with normal dogs and the previous observation by Zunz and Vesselovsky (1939) that administration of yohimbine resulted in an antidiuretic effect on water diuresis it was of interest to determine whether this antidiuretic action could be elicited in an animal which did not possess a functioning posterior lobe. Consequently four dogs with diabetes insipidus were obtained, the condition having been produced by surgical interruption of the hypothalamic stalk. These animals showed a marked and continuous polyuria.

The same experimental procedures were followed with the diabetes insipidus animals as with the normal dogs excepting that the water intake in some cases was not controlled while in other cases the amount of water administered equaled the average amount imbibed over a 2 week pre-experimental period. In all cases however the daily water intake was recorded.

When an animal was allowed water ad libitum and treated with yohimbine (2 mg./kg.) a marked increase in urine volume beyond that of the previous polyuria occurred. The water intake and urine volume increased while the specific gravity decreased (fig. 2).

These results suggest that the increased polyuria might be the direct result of the increased water intake on the part of the animal during the period of drug administration. Therefore the water intake was limited as described above and the experiments repeated. In these cases the polyuria did not increase but neither was there a significant antidiuretic effect (fig. 3).

It was thought that perhaps the absence of antidiuretic effects in diabetes insipidus animals might possibly be due to the polyuria

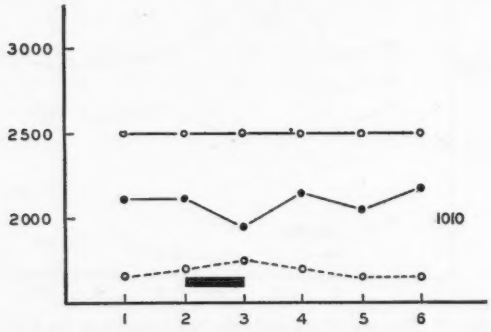

FIG. 3. Dog #9. With diabetes insipidus; wgt. 14.5 kg.
●——● Urine output; ○——○ Water intake; ○——○ Specific gravity; ▬ Period of administration of 2 mg. kg. of yohimbine HCl subcutaneously 3 times daily. Animal was restricted to average water intake over 2 week period. Note the absence of antidiuretic effect.

which is characteristic of these dogs. That is, the large volume of urine in some way may overshadow any renal effects yohimbine might produce. To test this possibility 2 diabetes insipidus animals were placed on a regime of pitressin tannate in oil to control the polyuria. When the urine volume assumed a relatively normal and constant level yohimbine was again administered. While the animal was under the influence of pitressin tannate, yohimbine not only had no antidiuretic effect, but the water intake and urine volume both increased (fig. 4). In other words the effect obtained is just the opposite to that observed in normal animals.

In order to test further the possibility that the results obtained were renal effects not mediated through the posterior pituitary, determinations of creatinine clearance were made on diabetes insipidus dogs before and during yohimbine administration. The results of these experiments gave no evidence which could be construed as evidence of a direct action of yohimbine on the kidney.

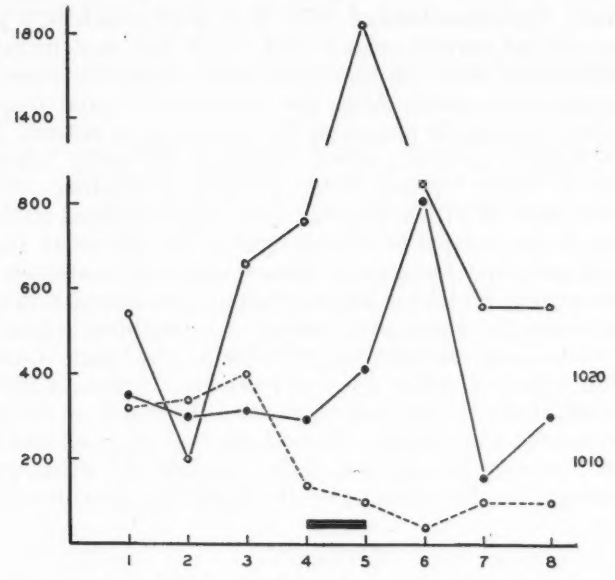

FIG. 4. Dog #6. With diabetes insipidus; wgt. 9.7 kg.

●———● Urine output; ○———○ Water intake; ○———○ Specific gravity; ▬▬ Period of administration of 2 mg. kg. of yohimbine HCl subcutaneously 3 times daily. This animal received 0.05 cc. of Pitressin Tannate in oil daily to control the polyuria throughout the experimental period. Note that yohimbine did not produce any antidiuretic effect in spite of the small urine output due to the antidiuretic effect of Pitressin Tannate.

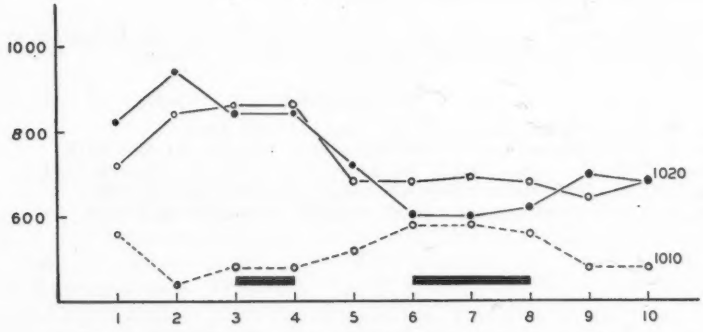

FIG. 5. Dog #2. Same animal as in graph 1 after complete hypophysectomy; wgt. 7 kilos.

●———● Urine output; ○———○ Water intake; ○———○ Specific gravity; ▬▬ Period of administration of 2 mg. kg. of yohimbine HCl subcutaneously 3 times daily. Note the absence of marked antidiuretic or diuretic action of yohimbine.

Completely hypophysectomized dogs. Two dogs which had previously been used as normal animals and which had demonstrated a typical antidiuretic effect on treatment with yohimbine were completely hypophysectomized. After the animals recovered from the operation they were again placed on the experimental regime. These animals exhibited a polyuria which remained distinctly below the level which would be realized if the posterior lobe alone were removed. When treated with yohimbine these dogs exhibited no significant change in the volume or specific gravity of the urine (fig. 5). This fact suggests that the greater diuresis observed in animals with a degenerated posterior lobe might be attributed to stimulation of the anterior lobe and the consequent release of some diuretic principle.

Attempts to assay antidiuretic principle in the urine of normal dogs treated with yohimbine by two methods (Ingram, Ladd and Benbow, 1939; Hare, Hickey and Hare, 1941) have not, so far, given satisfactory results. Possibly the amounts released are small and being used up in the body do not find their way into the urine, or the amounts released are too small to be discovered by present means of assay.

SUMMARY

It is shown that the alkaloid yohimbine when administered to normal dogs produces an antidiuresis which it is assumed results from the release of an antidiuretic principle by the posterior lobe.

When yohimbine was administered to dogs with transected posterior lobes, or completely hypophysectomized the polyuria is not relieved. On the contrary, in diabetes insipidus animals the urine volume increases suggesting the release of some diuretic principle from the anterior lobe. This presumption gains support from the observation that in completely hypophysectomized dogs no significant change in urine volume occurred.

I wish to express my appreciation to Doctors E. G. Gross, W. R. Ingram and K. Hare for valuable aid and interest in the course of this investigation.

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SOME QUANTITATIVE ASPECTS OF ESTROGEN ASSAYS

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METHODS for the assay of estrogens have been devised primarily for the measurement of the estrogenic potency of extracts of tissue and urine. The estimation of potency of commercial preparations of oil solutions of estrogens differs in several respects from similar determinations on such extracts. The commercial preparations are either solutions of crystalline materials or highly purified extracts; only a few contain estriol or its salts; all carry a declaration of potency which usually serves the purpose of a preliminary range-finding test, and the quantity of active material available is usually large. These facts allow a more precise comparison of the response to the unknown with the response to the standard. The result is a decreased error in the estimation of potency of the unknown.

There remains to be considered, however, *a*) the exact assay technique that is preferable; *b*) the accuracy that may be expected by using this technique; *c*) the animal of choice, rat or mouse; and *d*) which of the available standards is to be used as a reference material. The answer to these points is complicated by the multiplicity of derivatives of the naturally occurring estrogens appearing on the market and also by the development of synthetic estrogens, such as diethylstilbestrol, as substitutes for the natural products.

This report describes a biological assay method used by this laboratory, the statistical characteristics of the method as determined from assays conducted during 1941 and the first half of 1942, and preliminary experiments on mice designed to measure the variability that may be expected when this species is used for the assay.

MATERIALS

The assay data, using rats, were taken from routine determinations of potency of commercial preparations, but only that portion of the assay pertaining to the response to the particular standard used was tabulated for the purpose of this report. In this way variations in response from assay to assay have been evaluated. The data from 46 assays of International Standard Estrone using 2205 animals and 13 assays of International Standard Estradiol Benzoate using 687 animals are included. The assays using mice were of a preliminary nature and were designed to measure the variability of response in this species. They have been reported here because further work will not be possible for some time.

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Crystalline International Standard Estrone and Estradiol Benzoate were used exclusively. Alcoholic solutions of known concentrations were prepared and accurately measured volumes of these solutions were added to known volumes of corn oil. The oil was heated gently to remove the alcohol before injection.

All animals used in these experiments were of albino stock from breeding colonies maintained in this laboratory, and had not been inbred. They were housed in metal cages in air conditioned rooms, the temperature of which varied from 76 to 80°F. and the relative humidity from 50 to 65%. Feed consisted of commercial diet in pellet form supplemented with lettuce.

ASSAY METHODS

Rats. All rats appeared healthy before being transferred to the assay colony. They were selected and prepared for assay as described by Kahnt and Doisy (1928).

Two estrous responses were induced on successive weeks as a primary process, the first by an overdose of an aqueous solution of known estrogen (1.5 μg. of estrone) and the second by a dose slightly greater than the threshold dose of the same solution (1.2 μg. estrone). All test solutions whether dissolved in oil or water were injected subcutaneously on the back in 3 equal parts at 4½ hour intervals. An occasional animal failed to respond to both priming doses and was discarded as being too insensitive.

Rats prepared in this manner were used for routine assays of commercial preparations on alternate Mondays and on the intervening Mondays all animals were primed with an aqueous solution of estrogen (1.2 μg. of estrone). No animal was used for assay that had not responded positively to priming one week previously. The purpose of this procedure was two-fold: *a*) to standardize the immediate history of each animal on assay, and *b*) to provide adequate time for the removal of subcutaneous oil pockets. We have never been confronted with the complicating factor of accumulated subcutaneous deposits of oil when using this procedure.

Vaginal smears were made with dampened, cotton-tipped toothpicks 48, 52¼, 56½ and 72 hours after the first injection when unesterified estrogens were being tested. When estradiol benzoate was being assayed an additional smear made 76½ hours after the first injection insured adequate coverage of the time of maximum response. The time of maximum response for other esters would have to be determined and smears taken at the proper time.

All the smears from a single animal were placed on different sections of the same glass slide which was identified by the designation of the animal. The slides were stained with hematoxylin and eosin before microscopic examination and evaluation. Any smear in which the majority of the cells were cornified epithelium was regarded as a positive smear. The reaction of the animal was rated as positive if any one of the smears was positive. All smears were read by one individual.

The group of rats available for an assay were divided into 6 sub-groups, 3 of which received the standard at 3 dosage levels and the remaining 3 sub-groups received the unknown at 3 dosage levels. An effort was made to adjust the dosage levels of both the standard and the unknown so that about 15% of the animals responded to the lowest level, about 50% to the middle level and about 85% to the highest level. This ideal distribution was seldom achieved but data were always available that allowed a differentiation to be made be-

tween doses, based on the physiologic response of the assay animals. The sensitivity of the assay method is determined from these data.

The influence of the weight of the animals upon their response to a given dose of estrogen has been examined. Weight variation not greater than ± 50 gm. from the mean weight of the group (235 gm. for the rats used in these assays) had no influence on the sensitivity of the group. In order to keep the variations in weight within these limits the food intake of each animal was restricted to 12 to 14 grams per day.

Mice. Mice were ovariectomized when about 3 months old and the completeness of the operation was checked in the usual manner. They were then primed with an aqueous solution of known estrogen (0.6 μg. of estrone) administered subcutaneously in 3 equal doses at 4½ hour intervals. One week later those which responded positively were ready for use in an assay. When oil solutions were assayed with mice it was found necessary to allow more time for the removal of subcutaneous deposits of the oil than was necessary with rats. Hence in these cases the mice were used for assay only once every 3 weeks. One week before an assay was to be performed all mice were primed as above and only those that showed estrous smears were used. The group of mice available for assay was divided into sub-groups as described for rats. Oil solutions for assay were administered in a single dose in volumes of 0.1 cc. and 0.2 cc.

RESULTS

International Standard Estrone—Rats. The analysis of the results of these assays was based on the observation that when dosage and percentage response are converted to suitable functions, namely, logarithm-dose and probits,[1] the relationship between them can be expressed by a straight line. From this line the response to any given dose may be approximated. For a given slope the error of this approximation depends upon how closely the observations fit the straight line. In other words the approximation has a certain standard error of estimate which can be calculated from the data. The methods used for the calculations are described by Miller, Bliss and Braun (1939). Table 1 shows the data from 3 assays as samples chosen at random from the 46 assays reported here. The calculated slopes (b) of the 3 straight lines are given in the fourth column and the estimated logarithim (dose $\times 10$) for a 50 percent response ($\log 10ED_{50}$) in the sixth column. In the fifth column is given σ_b, the standard error of the slope, and in the seventh column, $\sigma_{\log}$ ($10ED_{50}$), the standard error of the log (dose $\times 10$) for a 50 percent response.

The calculated results for all 46 assays are given in table 2. The data from all assays were combined and tested in 2 distinct ways.

[1] Statistical tables expressing the area under the normal bell-shaped curve in terms of standard deviation units are given in most statistical texts. Probits are units derived from this normal bell-shaped curve by using these tables. If the total area under the normal bell-shaped curve be considered as representing 100% response, any lesser percentage response can be expressed as a proportionate part of the total area. The number of probits equals the number of standard deviation units (corresponding to the proportionate part of area as given in the table) plus five to avoid negative numbers.

The first test involved the homogeneity of the slopes. The combined slope of the 46 determinations was calculated to be $7.553 \pm .488$ with a range of individual values of from 2.152 to 21.562. The chi-square test applied to these data resulted in a value of $X_{bc}^2 = 57.11$ ($N = 46$), and in the conclusion that the slopes were homogeneous within the limits of sampling error. Figure 1 shows the variation of slope with

TABLE 1. SAMPLE INDIVIDUAL ASSAY DATA AND THEIR STATISTICAL CHARACTERISTICS

Date	Dose μg	Response	Slope b	σ_b	Log(10ED$_{50}$)	$\sigma_{\text{Log}(10\text{ED}_{50})}$	ED$_{50}$ μg
2- 3-41	.969	9/17	4.1851	3.017	.9888	.0370	.975
	1.162	14/17					
	1.387	12/16					
11- 3-41	.969	4/19	12.4501	3.241	1.0605	.0260	1.149
	1.162	4/16					
	1.387	15/17					
7-20-42	.969	2/15	8.9319	3.372	1.1287	.0356	1.134
	1.163	4/15					
	1.387	9/15					

time. No seasonal trend exists, in agreement with Emmen's (1939) results on mice, nor is there any tendency for a consistent trend in values either up or down. The authors can ascribe no reason for the differences in the slopes observed. It appears to be a characteristic of the animals and as shown as the chi-square test the slopes were not significantly different.

The second test involved the homogeneity of the assays as a whole considering simultaneously the slopes and the values of log (10ED$_{50}$). Since the log (10ED$_{50}$) values are dependent on the slope they cannot be tested independently for homogeneity. In this case the analysis of variance technique was used wherein a function of the sums of the squares of the distances from the observed points to the calculated regression line (error of estimate) in any one assay was compared with the variation between assays.[2] Tested in this manner the assays were not homogeneous (Snedecor's $F = 1.87$; $P < .01$). Figure 2 shows the values of log (10ED$_{50}$) plotted against time. The average log (10ED$_{50}$) value is shown by the horizontal line. A significant trend is indicated by the curved line which shows a gradual increase and then a decrease in the log (10ED$_{50}$) values over the period covered by the 46 assays.

It may be concluded from this analysis of estrone assays that it is necessary to compare the response to an unknown with the response to the standard determined simultaneously and that if quantitatively accurate results are to be expected, the assay must be so designed that the slope and position of the regression lines may be determined.

[2] The original data of all experiments were considered as one total experiment for the purpose of fitting a line and obtaining residuals from that line. An analysis of variance was performed with the sum of squares of the residuals from this line as the "total" and using the pooled sum of squares of the residuals around the respective lines fitted to each individual experiment as the "within." The "between" is calculated by subtraction. See any statistical text on Analysis of Variance and Covariance.

TABLE 2. THE STATISTICAL CHARACTERISTICS OF THE RESPONSES OF RATS TO INTERNATIONAL ESTRONE IN 46 ASSAYS

Date	Slope (b)	σ_b	Log ($10ED_{50}$)	$\sigma_{\log (10ED_{50})}$	ED_{50} μg
12- 9-40	2.857	2.779	1.0649	.0236	1.161
12-24-40	10.618	3.344	1.0205	.0325	1.048
1- 6-41	10.316	3.862	.9492	.0529	.890
1-21-41	6.093	3.141	.9845	.0390	.965
2- 3-41	4.185	3.017	.9888	.0370	.975
2-10-41	7.326	3.364	1.0197	.0322	1.046
2-17-41	10.205	3.226	1.0243	.0286	1.058
3- 3-41	21.562	4.384	1.0280	.0326	1.067
3-31-41	13.237	3.532	1.0146	.0317	1.034
4-21-41	2.698	2.713	1.0880	.0243	1.225
4-28-41	6.492	2.611	1.0083	.0271	1.019
5- 5-41	12.669	4.085	1.0267	.0346	1.063
5-26-41	3.486	3.377	1.0489	.0291	1.119
6- 9-41	6.710	2.849	1.0961	.0259	1.248
6-16-41	4.797	2.977	1.0380	.0260	1.091
6-23-41	7.199	3.632	.9803	.0382	.956
6-30-41	7.249	2.915	1.0673	.0239	1.168
7- 7-41	10.213	3.739	1.0806	.0285	1.204
7-21-41	2.747	2.694	1.0228	.0265	1.054
7-28-41	8.859	2.973	1.0417	.0248	1.101
8- 4-41	8.464	2.947	1.0941	.0270	1.242
8-11-41	10.079	3.400	1.0445	.0278	1.108
8-18-41	4.736	2.918	1.0420	.0257	1.102
9- 8-41	8.569	3.853	.9378	.0612	.867
11- 3-41	12.450	3.241	1.0605	.0260	1.149
11-24-41	6.032	2.993	1.1289	.0344	1.346
12-15-41	13.257	3.572	1.0239	.0296	1.057
1-12-42	7.669	2.828	1.0860	.0251	1.219
1-19-42	8.663	3.097	1.0567	.0260	1.139
1-26-42	15.459	3.561	1.0622	.0275	1.154
2- 2-42	2.152	3.125	1.0082	.0328	1.019
2- 9-42	4.764	2.786	1.1263	.0310	1.338
2-16-42	4.626	3.174	.9772	.0405	.949
3- 9-42	6.521	3.192	1.0378	.0275	1.091
3-30-42	8.277	3.029	1.0247	.0278	1.059
4-20-42	6.939	3.452	1.0622	.0282	1.154
4-27-42	3.236	3.426	.9383	.0547	.868
5- 4-42	17.843	4.787	.9866	.0427	.970
5-11-42	10.528	3.466	1.0177	.0315	1.042
5-18-42	5.895	3.885	.9944	.0378	.987
5-25-42	5.196	2.961	1.0677	.0245	1.169
6- 1-42	9.958	3.676	.9670	.0462	.927
6- 8-42	8.685	3.112	1.0572	.0251	1.141
6-15-42	9.246	4.926	.9273	.0711	.846
6-22-42	11.978	4.373	.9530	.0556	.897
7-20-42	8.932	3.372	1.1287	.0356	1.345
$N=46$	$b_c=7.553$		wtd. ave. $=1.0454$		1.110

International Standard Estradiol Benzoate—Rats. Similar analyses of 13 assays (Table 3) using estradiol benzoate gave a value for the combined slope (b_c) of $9.612 \pm .863$ with a range of individual values from 7.37 to 11.785. A chi-square test of these 13 slopes showed that they were homogeneous ($X_{b_c}^2 = 2.124$; $N = 13$). The variance analyses of the errors of estimate showed these 13 assays to be heterogeneous (Snedecor's $F = 6.77$; $P < .01$) as was true in the case of estrone. There was no evidence of any trend with time in the values of log ($10ED_{50}$).

Comparison of the results of analyses of the assays of estrone with

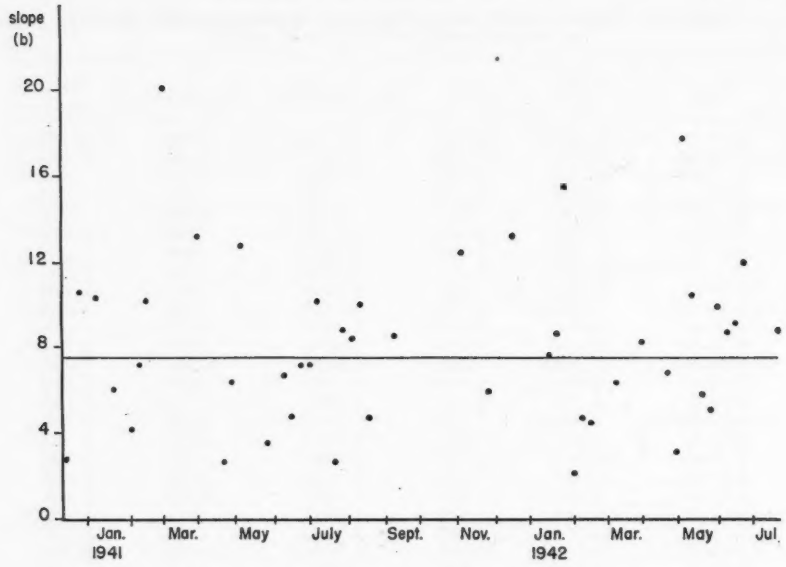

FIG. 1. Variation of slope (b) according to time assay was made.
—International Standard Estrone—Rats.

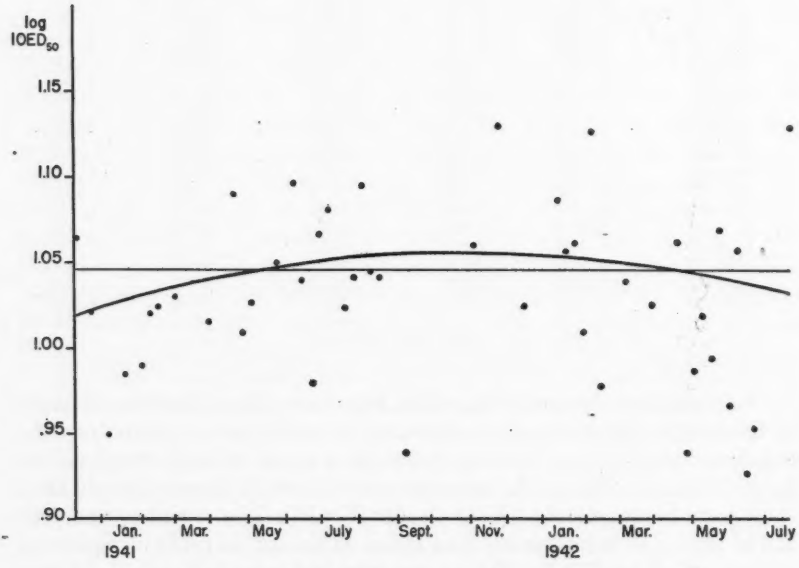

FIG. 2. Variation of log 10 ED_{50} according to time assay was made.
—International Standard Estrone—Rats.

those of estradiol benzoate showed that they were not essentially different. The combined slope for estrone does not differ "very significantly" from that calculated for estradiol benzoate in spite of the fact that the two substances are different chemical compounds and the time interval between injection and response was different. Furthermore, the groups of assays of the two substances were heterogeneous among themselves. From a comparison of errors of estimate

TABLE 3. THE STATISTICAL CHARACTERISTICS OF THE RESPONSE OF RATS TO INTERNATIONAL STANDARD ESTRADIOL BENZOATE ON 13 ASSAYS

Date	Slope b	σ_b	Log ($10ED_{50}$)	$\sigma_{Log\ (10ED_{50})}$	ED_{50} μg
9-23-39	11.472	3.912	.2889	.0277	.194
10- 2-39	8.223	3.376	.3044	.0249	.202
12- 9-40	10.051	3.274	.2934	.0220	.196
12-24-40	8.867	4.149	.2242	.0413	.168
2-24-41	11.653	3.920	.2077	.0304	.161
2-24-41	10.282	3.414	.3584	.0196	.228
3-10-41	9.931	3.795	.2808	.0276	.191
3-10-41	7.371	3.270	.2655	.0192	.184
3-24-41	9.145	1.763	.3510	.0171	.224
4-21-41	8.183	2.975	.3605	.0204	.229
5-19-41	11.785	3.356	.2996	.0221	.199
9-27-41	9.542	3.047	.3196	.0201	.209
10- 6-41	10.973	3.281	.2984	.0217	.199
Ave.	$b_c = 9.612$		wtd. ave. = .3090		.204

within assays it might be expected that the accuracy of the estradiol benzoate assays would be greater than that of estrone.

Only 3 of the assays included were designed to measure the relative potency of estrone and estradiol benzoate. However, a comparison of the weighted average ED_{50} values as reported gave a ratio of potencies of 5.45 to 1. The 3 assays designed to test this ratio resulted in values of 5.404, 5.486 and 6.126 and the weighted average of the 3 was 5.58 to 1. It is necessary to point out that these values are to be considered valid only under the specific conditions of assay that exist in this laboratory and do not apply to determinations of the ratio in other animals or in man.

International Standard Estrone—Mice. The analysis of the results of 6 assays of estrone on mice using a single dose of 0.1 cc. of oil showed the slopes to be homogeneous (by means of the chi-square test) with a combined slope (b_c) of $2.713 \pm .4781$ and a range of from 1.634 to 4.075 for the slopes of individual assays. It will be noted that this b_c value was definitely smaller than that obtained for rat assays. Hence, other things being equal, a less accurate estimate of ED_{50} would be expected from the mouse assay than from the rat assay since the standard error of ED_{50} is inversely proportional to the slope.

In another series of 7 assays of estrone on mice, 0.2 cc. of oil was used as the solvent instead of 0.1 cc. A statistical analysis of these results showed that the slopes were not homogeneous although they gave a satisfactory combined slope, $b_c = 7.790 \pm .7740$. The amount of

data in either series was not considered sufficient to warrant further statistical analysis.

Although these series were not designed for the purpose of comparison and the results on mice are not entirely satisfactory, some tentative conclusions can be drawn. The sensitivity of an assay, as measured by the slope of the dosage-response curve, was of a different order of magnitude for estrone in 0.1 cc. of oil than for estrone in 0.2 cc. Also the apparent potency of the estrone is greater when the larger volume of solvent is used. The unweighted average ED_{50} for 0.1 cc. of oil solvent was 0.109 μg. $\pm$.0247 μg. and for 0.2 cc. was 0.064. $\pm$.0022 μg. These results seem to indicate 2 apparent potencies of the same material: 9 million mouse units per gram and 16 million mouse units per gram, when measured under different conditions of assay.

International Standard Estradiol Benzoate—Mice. Two separate similar series of assays as described for estrone using different volumes of solvent (0.1 cc. and 0.2 cc.) were performed using estradiol benzoate. The slopes of the regression lines were homogeneous for each of the two volumes of solvent:

0.1 cc.

$b_c = 7.011 \pm 1.080$ with a range of the 5 individual values from 11.244 to 5.094.

0.2 cc.

$b_c = 5.822 \pm 1.397$ with a range of the 4 individual values from 7.038 to 3.244.

As in the estrone group the data did not seem to warrant further statistical analysis. The apparent potency of estradiol benzoate was about 20 million mouse units per gram under both sets of conditions. ($ED_{50} = 0.047\mu g. \pm .0034\mu g.$ for 0.1 cc. of oil and $0.051\mu g. \pm .0045\mu g.$ for 0.2 cc. of oil.)

On the basis of results available in this series it is not advisable even to attempt to place a tentative value on the relative potency of estrone and estradiol benzoate as measured in mice, but merely to point out that the ratio of potencies probably differs in mouse assays from that in rat assays.

DISCUSSION AND CONCLUSIONS

The accuracy which can be reliably attributed to any assay procedure is one of the principal factors that determines its usefulness. Assays designed as outlined above allow an evaluation of many of the variables and an estimation of the accuracy of each assay. The estimation of potency of commercial estrogenic preparations conducted in this manner usually had a calculated error not greater than $\pm 15\%$ (99 times out of 100) using groups of 15–20 rats on each dosage level. This definition of the limits of error of any assay is an expression of the confidence that may be placed in the results obtained in any one laboratory.

The choice of animal to use for estrogenic assays, rat or mouse,

depends on the variables characteristic of the respective species and on the accuracy that may be expected of the determinations. The extensive results published by Emmens (1929) and Palmer (1941) on estrogenic assays using mice are quite similar to ours. The slopes (b) of the regression lines of the assays of each of these authors as well as those reported here were in each case less steep for mice than for rats. Furthermore, it has not been shown that the slopes of repeated assays with mice are homogeneous for any one colony. Palmer, in fact, concluded that his results are not homogeneous. These observations make the interpretation of comparative assays using mice, even within a single laboratory, difficult and of doubtful significance. On the other hand, it has been shown that rats are not suitable test animals for the assay of estriol or its salts (Curtis, Miller and Witt 1937). Until further evidence on the reliability of assay results obtained on mice is available, rats appear to be the animal of choice for the assay of commercial estrogenic preparations with the exception of those containing estriol.

The standards of physiological potency in use at the present time are the International Standard Estrone or Estradiol Benzoate or their U.S.P. counterparts. These physical standards are entirely satisfactory but the definition of the units together with the absence of a recognized method of assay make the use of these standards unsatisfactory for the following three reasons:

1. The units of potency of the standard are defined in identical terms on the basis of mass without any relation to or consideration of the difference in the capability of the two compounds to cause a physiological change.

2. The potency of new derivatives and of new synthetic estrogenic compounds cannot be evaluated if one adheres strictly to the limitations of a satisfactory assay with regard to time of response as imposed by the monograph defining the standards and units.

3. The relative strength of different estrogenic compounds is not clearly expressed by relating mass units (mg) or by relating biological International units.

It is suggested that these 3 objections may be resolved by adopting either multiple standards (i.e., a standard for each new compound) or a single standard with which all estrogenic compounds may be compared. At the present time it is the practice in this laboratory to use a combination of single and multiple standards. All unesterfied commercial preparations and all unpurified mixtures of naturally occurring estrogens are compared with the Standard Estrone, and all esterified estrogens are compared with the Standard Estradiol Benzoate. Since the length of time from the initial injection to the response is characteristic for each different compound, such comparisons of potencies are satisfactory only if the reactions of the animals are observed at the time of their maximal response. Such assays do not conform in all cases to the limitations of a valid assay as described

by the Health Organization of the League of Nations where it is required that the response to the standard and the unknown must occur in the same 24-hour period. The procedure, of course, satisfies only one of the objections previously listed.

It may be desirable, particularly from the point of view of the assayist, to establish multiple standards. This would require a standard estrone, estradiol, estradiol benzoate, estradiol di-benzoate, stilbestrol, etc., each with an individual unit, so defined that their relative potencies would be known and if possible equal. Fortunately, the nature of the estrogen in each commercial preparation is declared on the label and from this information the suitable standard for comparison can be chosen. On the other hand, it may be desirable to adopt a single standard in which case the difficulties of assay still remain but would be no greater than under the present conditions. To make a single standard effective it would be necessary to adopt a rigidly prescribed assay procedure patterned after methods for other preparations now described in the U. S. Pharmacopoeia so that:

1. The method measures threshold doses only.
2. The response of any group of assay animals is observed at the time of maximal reaction which is determined for each estrogen under the conditions of the adopted assay method. It should be generally recognized that esterification of an estrogenic compound delays and prolongs the expected physiological response.
3. Each unknown is assayed by simultaneous comparison with the standard.
4. The relative potencies of different estrogenic compounds are applicable only to the animal in use under the conditions defined. They may or may not be the same in humans. As more information becomes available concerning the relative potencies in humans the assay method might be revised.

SUMMARY

A design for the assay of commercial estrogens using rats is described. The statistical characteristics of a series of 46 such assays including the response of 2205 rats is presented. The accuracy of the method is about $\pm 15\%$ (99 times out of 100) using 15–20 animals for each of 3 dosage levels. Assays using mice do not seem to have a similar degree of accuracy.

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ON THE IN VITRO ACCUMULATION OF INORGANIC IODIDE BY SURVIVING THYROID TISSUE WITH RADIOACTIVE IODINE AS INDICATOR[1]

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THE EARLY WORK of Marine (1915; Marine and Feiss, 1915) as well as the more recent observations with radioactive iodine (Hamilton and Soley, 1939; Hertz, Roberts, Means and Evans, 1940; LeBlond and Sue, 1940; Perlman, Morton and Chaikoff, 1941) leaves no doubt that the thyroid gland possesses an extraordinary capacity for the rapid removal of large amounts of circulating iodide. This iodine-concentrating capacity of thyroid tissue has not as yet received a satisfactory explanation. To obtain information on the mechanism involved, an investigation has been made here of the uptake of iodine and its subsequent conversion to thyroxine and diiodotyrosine by surviving thyroid slices in the presence of cyanide, sulfide, azide and sulfanilamide. It was shown earlier that these compounds inhibited the formation of thyroxine and diiodotyrosine by thyroid tissue, the first 3 by interfering with the cytochrome-cytochrome oxidase system (Schachner, Franklin and Chaikoff, 1943), the sulfanilamide by interfering in a manner as yet unidentified (Franklin and Chaikoff, 1943). The results obtained here show that, under certain conditions, the iodine-concentrating capacity of thyroid tissue cannot be accounted for by the conversion of inorganic iodide to thyroxine and diiodotyrosine.

EXPERIMENTAL

Preparation of Solutions: The bicarbonate-Ringer's solution was prepared as described by Krebs and Henseleit (1932). It was saturated with a gas mixture consisting of 5 per cent CO_2 and 95 per cent O_2. The radioactive iodide (I^{131}) was added to the bicarbonate-Ringer's solution in an isotonic solution of NaCl; the volume of the latter was always small in comparison with that of the Ringer solution used. Three cc. portions of this radioactive solution were transferred to 25 cc. Erlenmeyer flasks.

Solutions of the inhibitors were prepared of such a strength that, when 0.1 cc. of them was added to the 3.0 cc. of the radioactive bicarbonate-Ringer solution, the concentrations of the inhibitors recorded in Tables 4–7 were ob-

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tained. The effect of the addition of this small volume on the tonicity of the final medium was considered negligible.

The azide and cyanide were added as solutions of NaN_3 and NaCN. The sulfide solution was prepared as described elsewhere (Schachner, Franklin and Chaikoff, 1943). The above solutions as well as that of sulfanilamide were adjusted to pH 7.4 before their addition (in 0.1 cc. volume) to the reaction flasks.

No I^{127} was added to the incubation media used in the experiments of Tables 1, 2, 4, 5 and 6. The only I^{127} contained in the media of these experiments was that due to impurities in the reagent quality chemicals used in the preparation of the bicarbonate-Ringer medium.

I^{127} was added in experiments of Tables 3 and 7. The amounts recorded were added to the medium as 0.1 cc. solution of KI.

Preparation of Slices: The tissues were obtained from sheep and beef that had been sacrificed by exsanguination at the local abattoir. They were wrapped in cellophane and packed in ice immediately after their excision from the body and kept so until prepared for slicing. The technique used in the preparation of the tissue slices has been described elsewhere (Morton and Chaikoff, 1943). Approximately one hour elapsed between the death of the animal and the time the slicing of the tissues was begun.

Three hundred mg. of tissue slices were transferred to each reaction flask containing the prepared medium. The atmosphere above the liquid was then displaced with a gas mixture consisting of 5 per cent CO_2 and 95 per cent O_2. The flask was then tightly stoppered and incubated at 38°.

Separation of Slices from the Medium: In previous studies the contents of the entire flask, i.e. slices as well as medium, were analyzed for thyroxine, diiodotyrosine and inorganic iodide. The present investigation was concerned primarily with the accumulation of iodine by the slice. It was therefore necessary to effect a separation of the thyroid slices from their surrounding medium. This was done as follows. The solution in the flask was decanted off, leaving the slices. They were then removed from the reaction flask and placed in a Syracuse watch glass containing 3.0 cc. of non-radioactive bicarbonate-Ringer solution (Krebs-Henseleit). After about 20 seconds the slices were lifted out of the solution and transferred to another Syracuse dish containing 3 cc. of fresh non-radioactive bicarbonate-Ringer solution. The slices were kept in the latter for about 20 seconds and then placed on a filter paper moistened with bicarbonate-Ringer solution, in order to remove excess liquid adhering to the surface of the slices. The slices were then weighed; during the incubation and subsequent washing, the thyroid slices lost approximately 10 per cent of their initial weight. Care was taken to ensure that the slices remained intact throughout the procedure.

Fractionation of I^{131} in Slices: The slices were transferred to a 25 cc. Erlenmeyer flask containing 6 cc. of 2 N NaOH and hydrolyzed for 8 hours on a steam bath. The hydrolysate was transferred to a separatory funnel and 0.1 cc. of 0.2 M KI added as carrier. The solution was then made acid to methyl orange by the addition of 18 N H_2SO_4. One cc. of 0.02 M KIO_3 was added at once and the I_2 formed by the oxidation of iodide extracted immediately with carbon tetrachloride. Three 10 cc. portions of this solvent were used to remove the I_2. The completeness of extraction was indicated by the disappearance of the purple color.

The carbon tetrachloride fractions were combined and the I_2 contained

in them reduced to iodide by the addition of 1.0 cc. of 0.1 N $Na_2S_2O_3$. The iodide formed was then extracted with three 10 cc. portions of water and these aqueous extracts (which contained the inorganic iodide I^{131} of the hydrolysate) combined.

The iodine that remained in the acidified aqueous solution after it had been extracted with carbon tetrachloride contained the iodine that was not converted to I_2 by treatment with iodate. The I^{131} so obtained is referred to here as organic.

Determination of Radioactivity: Aliquots of the inorganic I^{131} and of the organic I^{131} were transferred to Coors milk ashing dishes (12×45 mm.) containing a small piece of lens paper and evaporated to dryness on a hot plate. A few drops of 2 N NaOH were added to prevent loss of iodine by volatilization. The lens paper served to promote an even distribution of the dried salt. Radioactivity was measured by means of a scale-of-8 Geiger-Müller counter. The counting tube consisted of a glass cylinder 64 mm. in diameter containing a central tungsten wire, an internal copper cylinder and a thin mica (4.83 mg. per sq. cm.) window supported by a brass grid. The tube was filled with a gas mixture composed of 10 per cent alcohol and 90 per cent argon at 4 cm. pressure.

RESULTS

The iodine-concentrating capacity of slices of thyroid gland, liver, and spleen is shown in Table 1. As much as 85 per cent of the I^{131} of the Ringer's medium was taken up by 300 mg. of sheep thyroid slices during the course of one hour. In 2 hours a similar amount of beef

TABLE 1. THE ACCUMULATION OF I^{131} IN SURVIVING LIVER, SPLEEN AND THYROID SLICES
(300 mg. of slices were incubated at 38° in a bicarbonate-Ringer medium containing inorganic iodide I^{131}. No I^{127} was added.* The slices were then separated from the medium and analyzed.)

Expt. No.	Tissue	Time	Per cent of Ringer's I^{131} recovered in Slices as			Per cent of I^{131} within the slice that was organically bound
			Organic†	Inorganic	Total	
1	Sheep liver	1 hr.	0.1	2.6	2.7	
	Sheep liver	1 hr.	0.0	2.1	2.1	
	Sheep liver	1 hr.	0.1	2.2	2.3	
	Sheep thyroid	1 hr.	57.5	21.5	79.0	73
	Sheep thyroid	1 hr.	56.9	23.1	80.0	71
	Sheep thyroid	1 hr.	63.4	21.9	85.3	74
2	Sheep liver	1 hr.	0.3	4.0	4.3	
	Sheep liver	1 hr.	0.3	3.9	4.2	
	Sheep liver	1 hr.	0.3	2.8	3.1	
	Sheep thyroid	1 hr.	45.6	33.6	79.2	58
	Sheep thyroid	1 hr.	61.0	18.6	79.6	77
	Sheep thyroid	1 hr.	51.2	24.7	75.9	68
3	Beef spleen	2 hrs.			1.5	
	Beef spleen	2 hrs.			1.4	
	Beef thyroid	2 hrs.			87.6	
	Beef thyroid	2 hrs.			88.2	

* The only I^{127} contained in the reaction flasks was that derived from the reagent quality chemicals used in the preparation of the media.
† Refers to thyroxine-like and diiodotyrosine-like I^{131} (see text).

TABLE 2. THE EFFECT OF TIME ON THE UPTAKE OF RINGER'S I^{131} BY THYROID SLICES
(300 mg. of sheep thyroid slices were incubated at 38° in a bicarbonate-Ringer medium containing inorganic iodide I^{131}. No I^{127} was added.* The slices were then separated from their medium and analyzed.)

Time of incubation, Min.	2	10	30	60	120
Per cent of Ringer's I^{131} recovered in thyroid slices.	3.7 3.6	41 39	61 62	69 64	79 62

* See footnote Table 1.

thyroid slices accumulated 88 per cent of the I^{131} added to the Ringer's solution. That surviving slices of thyroid tissue retain their extraordinary capacity for accumulating iodine is well brought out by comparing the above values with those obtained when slices of liver and spleen were incubated in a Ringer's medium containing I^{131}. Less than 4 per cent of the I^{131} was recovered in 300 mg. of liver slices in one hour, whereas less than 2 per cent of the Ringer's I^{131} was taken up by spleen slices in two hours.

The rate of uptake I^{131} by surviving thyroid slices is shown in Table 2. The most rapid uptake occurred during the first 10 minutes. By the end of 30 minutes, approximately 60 per cent of the radioactive iodine of the Ringer's medium had entered the slices.

Not all of the I^{131} accumulated by the thyroid slices is organically bound. Approximately 25 per cent of the I^{131} recovered in slices that had been incubated for one hour was still present as inorganic iodide (Table 1).

In the experiments described above, no I^{127} was added to the Ringer's medium; the traces of I^{127} present in the reaction flask were due to the impurities of the reagents used in the preparation of the Ringer's solution. In the experiments shown in Table 3, 20 micrograms of I^{127} (as potassium iodide) were added to the Ringer's solution

TABLE 3. ABSOLUTE AMOUNTS OF I^{127} ACCUMULATED BY THYROID SLICES
(300 mg. of sheep thyroid slices were incubated at 38° for one hour in a bicarbonate-Ringer medium containing inorganic iodide I^{131}. The slices were then separated from their medium and analyzed.)

Iodide I^{127} added to flask	Per cent of Ringer's I^{131} recovered in Slices as			Micrograms of Ringer's I^{127} incorporated into Slices as		
Micrograms	Organic†	Inorganic	Total	Organic†	Inorganic	Total
None*	67.3	20.7	88.0			
None*	46.9	35.0	81.9			
None*	67.6	21.1	88.7			
20.0	10.0	55.0	65.0	2.0	11.0	13.0
20.0	9.9	52.0	61.9	2.0	10.4	12.4
20.0	9.0	48.2	57.2	1.8	9.6	11.4

* No I^{127} was added to these flasks. The only I^{127} contained in the reaction flask was that due to impurities in the reagents used for the preparation of the medium.
† See footnote Table 1.

TABLE 4. THE EFFECT OF CYANIDE ON THE ACCUMULATION OF I^{131} BY THYROID SLICES
(300 mg. of sheep thyroid slices were incubated at 38° for one hour in a bicarbonate-Ringer medium containing inorganic iodide I^{131}. No I^{127} was added.* The slices were then separated from the medium and analyzed.)

Expt. No.	Compound added to Ringer Solution	Per cent of Ringer's I^{131} recovered in Slices as		
		Organic†	Inorganic	Total
1	none (control)	59.3‡	22.2‡	81.5‡
	cyanide 2×10^{-2} M	0.2	16.7	16.9
	cyanide 2×10^{-3} M	0.1	7.9	8.0
	cyanide 2×10^{-4} M	0.1	6.7	6.8
2	none (control)	52.6§	25.6§	78.2§
	cyanide 2×10^{-2} M	1.7	8.7	10.4
	cyanide 2×10^{-3} M	0.2	9.8	10.0
	cyanide 2×10^{-4} M	0.2	7.4	7.6

* See footnote Table 1.
† See footnote Table 1.
‡ These controls are the same as those used in experiment 1 of Table 1; hence only averages are given here.
§ These controls are the same as those used in experiment 2 of Table 1; hence only averages are given here.

in which the thyroid slices were suspended. This procedure permitted the determination of the absolute amounts of the Ringer's I^{127} that penetrated the thyroid slices. Table 3 shows that as much as 11.4 to 13.0 micrograms entered the thyroid slices in one hour.

Cyanide: Marine and Feiss first pointed out that cyanide inhibits the absorption of iodine by the perfused thyroid gland (1915). In a previous report from this laboratory, it was shown that the conversion of Ringer's I^{131} to radiothyroxine and diiodotyrosine is depressed by

TABLE 5. THE EFFECT OF SULFIDE ON THE ACCUMULATION OF I^{131} BY THYROID SLICES
(300 mg. of sheep thyroid slices were incubated for one hour at 38° in a bicarbonate-Ringer medium containing inorganic iodide I^{131}. No I^{127} was added.* The slices were then separated from the medium and analyzed.)

Expt. No.	Compound added to Ringer Solution	Per cent of Ringer's I^{131} recovered in Slices as		
		Organic†	Inorganic	Total
1	none (control)	48.6	26.5	75.1
	none (control)	57.2	16.9	74.1
	none (control)	54.9	21.2	76.1
	Sulfide 10^{-3} M	0.5	13.8	14.3
	Sulfide 10^{-3} M	0.6	16.7	17.3
	Sulfide 10^{-3} M	0.6	19.0	19.6
2	none (control)	60.6‡	25.6‡	86.2‡
	Sulfide 10^{-3} M	0.5	15.3	15.8

* See footnote Table 1.
† See footnote Table 1.
‡ The controls are the same as those used in Table 3; hence only averages are given here.

cyanide (Schachner, Franklin and Chaikoff, 1943). The results obtained here (Table 4) show that the impaired formation of thyroxine and diiodotyrosine observed under the influence of cyanide is associated with a decreased absorption of I^{151} by the slices. The addition of cyanide depressed the accumulation of I^{131} by the thyroid slices from control levels of approximately 80 to 7–17 per cent.

Sulfide: Table 5 shows that a concentration of 10^{-3} M sulfide depresses the uptake of Ringer's I^{131} by surviving thyroid slices as well as its conversion to thyroxine and diiodotyrosine.

Azide: The failure of thyroid slices to bind organically Ringer's I^{131} in the presence of 5×10^{-3} M azide is again shown by the results recorded in Table 6.

TABLE 6. THE EFFECT OF AZIDE ON THE ACCUMULATION OF I^{131} BY THYROID SLICES (300 mg. of sheep thyroid slices were incubated for one hour at 38° in a bicarbonate-Ringer medium containing inorganic iodide I^{131}. No I^{127} was added.* The slices were then separated from the medium and analyzed.)

Expt. No.	Compound added to Ringer Solution	Per cent of Ringer's I^{131} recovered in slices as		
		Organic†	Inorganic	Total
1	none (control)	53.6‡	21.5‡	75.1‡
	azide 5×10^{-3} *M*	1.2	52.3	53.5
	azide 5×10^{-3} *M*	1.1	45.6	46.7
	azide 5×10^{-3} *M*	1.3	42.4	43.7
2	none (control)	52.6§	25.6§	78.2§
	azide 5×10^{-3} *M*	1.2	52.3	53.5
	azide 5×10^{-3} *M*	1.3	45.6	46.9
	azide 5×10^{-3} *M*	1.1	42.4	43.5

* See footnote Table 1.
† See footnote Table 1.
‡ The controls are the same as those used in experiment 1 of Table 5; hence only averages are given here.
§ The controls are the same as those used in experiment 2 of Table 1; hence only averages are given here.

In the presence of 2×10^{-2} M cyanide (Table 4) and of 10^{-3} M sulfide (Table 5) the almost complete failure of thyroid slices to convert the Ringer's I^{131} to thyroxine and diiodotyrosine paralleled the reduction in the uptake of Ringer's I^{131} by the thyroid slices. Thus the cyanide reduced the amount of Ringer's I^{131} deposited in the thyroid slices from control values of 80 to 8 per cent (averages) and the amount of Ringer's I^{131} organically bound inside the thyroid slices from 56 to less than one per cent. Similarly sulfide reduced the penetration of Ringer's I^{131} from 78 to 17 per cent (averages) the amount organically bound from 55 to less than 1 per cent. In contrast to the parallelism noted above in the amounts of I^{131} organically bound and those taken up by the thyroid slices, azide led to a marked depression in the I^{131} organically bound but not in the amount that entered the tissue. Approximately 48 per cent of the Ringer's I^{131} was absorbed

by thyroid slices in the presence of 5×10^{-3} M azide; the amount organically bound, however, was reduced from 48 to approximately one per cent. In this case, therefore, the failure of the thyroid slices to convert the Ringer's I^{131} to thyroxine and diiodotyrosine cannot be ascribed to a failure in the absorption of I^{131} from the Ringer's medium.

Sulfanilamide: It has been demonstrated elsewhere that in the presence of 10^{-3} M sulfanilamide the conversion of Ringer's I^{131} to radiothyroxine and radiodiiodotyrosine is almost completely inhibited (Franklin and Chaikoff, 1943). It was also shown that when no I^{127} is added to the bath the penetration of Ringer's I^{131} into the thyroid

TABLE 7. THE EFFECT OF SULFANILAMIDE ON THE UPTAKE OF I^{131} AND I^{127} BY THYROID SLICES

(300 mg. of thyroid slices were incubated for one hour at 38° in a bicarbonate-Ringer medium containing inorganic iodide I^{131}. The slices were then separated from their medium and analyzed.)

Micrograms of Iodide I^{127} added to bath	Compound added to Ringer Solution	Per cent of Ringer I^{131} recovered in slices	Micrograms of Ringer I^{127} incorporated into slices
none*	none	91	
none*	none	73	
none*	none	92	
none*	10^{-3} *M* Sulfanilamide	63	
none*	10^{-3} *M* Sulfanilamide	68	
none*	10^{-3} *M* Sulfanilamide	68	
10.0	none	67	6.7
10.0	none	59	5.9
10.0	none	60	6.0
10.0	10^{-3} *M* Sulfanilamide	68	6.8
10.0	10^{-3} *M* Sulfanilamide	61	6.1
10.0	10^{-3} *M* Sulfanilamide	58	5.0

* No I^{127} was added to this flask. The only I^{127} contained in the reaction flask was that due to impurities in the reagents used in the preparation of the medium.

slices is not appreciably depressed. Since so little I^{127} was present in the Ringer's medium in the earlier experiments, it might be considered that the I^{131} uptake under this condition was not a satisfactory measure of the iodine-concentrating capacity of thyroid tissue. Hence in 6 of the reaction flasks of Table 7, 10 micrograms of I^{127} were added and the micrograms of I^{127} that entered the slices determined according to the precedure described for the experiments of Table 3. Table 7 shows clearly that 10^{-3} M sulfanilamide has little effect on the avidity of this glandular tissue for iodide. Thus, in the presence of the sulfanilamide, the 300 mg. of thyroid slices absorbed 5–7 micrograms of I^{127} from the Ringer's medium, whereas in experiments in which no sulfanilamide was added 6–7 micrograms of Ringer's I^{127} entered the thyroid slices.

DISCUSSION

Since nearly all of the iodine contained in the thyroid gland of the intact animal is present as either thyroxine or diiodotyrosine, it seemed possible that the rapid uptake of circulating iodine by the thyroid gland is due to a mechanism concerned solely with the conversion of inorganic iodide to these compounds. In order to test this view experimentally, the formation of thyroxine and diiodotyrosine at the expense of I^{131} of the medium has been compared with the total amount of I^{131} removed from the medium. If the binding of iodine as thyroxine and diiodotyrosine accounts for the extraordinary capacity of thyroid tissue to absorb iodine, it should be possible to depress the iodine-concentrating capacity of this tissue by various processes that inhibit the formation of thyroxine and diiodotyrosine.

The complete failure of the thyroid slices to convert Ringer's I^{131} to thyroxine and diiodotyrosine in the presence of the concentrations of cyanide and sulfide used here was accompanied by a very marked interference in the amounts of Ringer's I^{131} that entered the slices; hence the failure to observe the formation of radiothyroxine and radiodiiodotyrosine here could be due to an interference in the penetration of Ringer's I^{131} into the slices. But this mechanism cannot explain the effects of azide and more particularly those of sulfanilamide. Despite the almost complete suppression of the formation of thyroxine and diiodotyrosine from Ringer's I^{131} by azide, approximately 50 per cent of the Ringer's I^{131} had entered the slice. The dissimilarity in the amounts that entered the slice and in the amounts that were organically bound there is even more strikingly brought out in the case of sulfanilamide. At a concentration of 10^{-3} M this sulfonamide strongly suppressed the formation of radiothyroxine and radiodiiodotyrosine but had little effect on the iodine-concentrating capacity of this tissue. Since nearly all of the Ringer's I^{131} that was recovered in the slices in the presence of azide was present as inorganic iodide, it seems reasonable to conclude that the mechanism responsible for the iodine-concentrating capacity of thyroid tissue is separate and distinct from that involved in the conversion of inorganic iodide to thyroxine and diiodotyrosine.

These observations therefore bring to light 2 types of poisonous effects. One type is exemplified by the action of 2×10^{-2} M cyanide and 10^{-3} M sulfide; these inhibited penetration of the I^{131} into the thyroid slices and possibly the subsequent conversion of the iodide within the slice to thyroxine and diiodotyrosine. The other type of effect is shown by 10^{-3} M sulfanilamide; this compound depressed the formation of thyroxine and diiodotyrosine within the slice without, however, affecting appreciably the penetration of I^{131} into the slices.

The manner in which relatively large amounts of inorganic iodide are held by thyroid slices in the short intervals of these experiments

is not known at present. It is of interest to note that Lein has proposed that the early fixation of iodine in the thyroid gland of the intact animal involves an acetone-soluble form which he claims to be non-protein in nature (1943). He points out, however, that this initial fixation of iodine in an acetone-soluble form "will not account for the large quantities of iodine found within the thyroid."

SUMMARY

Surviving thyroid slices were incubated at 38° in a bicarbonate-Ringer solution containing radioactive iodide as indicator. The amounts of I^{131} that entered the slices were compared with the amounts of I^{131} organically bound within the slices.

Cyanide and sulfide, in addition to completely inhibiting the formation of thyroxine and diiodotyrosine, markedly depressed the accumulation of I^{131} by thyroid slices.

Despite the almost complete inhibition of thyroxine and diiodotyrosine formation by 10^{-3} M azide, as much as 60 per cent of the Ringer's I^{131} entered the slices.

Sulfanilamide, which strongly inhibited the *in vitro* conversion of Ringer's iodide to thyroxine and diiodotyrosine, had little effect on the iodine-concentrating capacity of thyroid tissue.

It is concluded that in thyroid tissue there exists a mechanism for concentrating iodine that does not depend upon the conversion of inorganic iodide to thyroxine and diiodotyrosine.

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EFFECT OF THIOURACIL AND THIOUREA ON THE THYROID GLAND OF THE CHICK[1]

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IT IS WELL ESTABLISHED that certain compounds which are derivatives of either aniline or thiourea will prevent the formation of thyroxine by the thyroid gland, and as a result the thyroid hypertrophies due to the compensatory action of the thyrotropic hormone of the anterior pituitary gland. The usual symptoms of hypothyroidism then become evident. Mackenzie, Mackenzie and McCollum (1941) first observed thyroid hypertrophy and hyperplasia in rats fed sulfaguanidine. The Mackenzies (1942) later reported that this thyroid change could not be prevented by iodine administration, but could be prevented by the administration of thyroxine. Phenylthiourea (Richter and Clisby, 1942), allythiourea (Kennedy, 1942) as well as many other aniline and thiourea derivatives (Mackenzie and Mackenzie, 1943; Astwood, Sullivan, Bissell and Tyslowitz, 1943; and Astwood, 1943a) have been shown to cause this thyroid enlargement and hyperplasia. Mackenzie and Mackenzie (1943) and Astwood, *et al.* (1943) have made detailed studies on the mechanism of the action of these thyroid inhibiting compounds.

Thiourea and thiouracil (Astwood, 1943b) and thiouracil (Williams and Bissell, 1943) have been used clinically with good results in the treatment of hyperthyroidism in man. Dempsey and Astwood (1943) have administered thiouracil and thyroxine simultaneously to groups of rats kept at different environmental temperatures, and the amount of thyroxine required to maintain normal thyroid weight at the various environmental temperatures was considered to be a measure of the actual thyroxine output of normal animals at such temperatures.

The activity of these thyroid-inhibiting compounds has been most widely studied in the rat. Mackenzie and Mackenzie (1943) studied the action of some sulfonamides and thiourea in the mouse, positive results being secured; they also reported that sulfaguanidine was effective in each of two young dogs. The results were negative in the guinea pig using sulfapyridine and sulfaguanidine. There

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was no thyroid inhibition in 2-week and 12-week-old chickens fed 2 or 3 per cent sulfaguanidine for varying lengths of time and the opinion was expressed that the chicken represents a true species difference as regards its thyroid response to sulfaguanidine.

The negative results secured by Mackenzie and Mackenzie with the chicken (1943) and the extensive study by Astwood (1943a) on the chemical nature and especially the potency of compounds which inhibit thyroid activity suggested that further experiments on thyroid inhibition in the chicken were desirable.

METHODS

Except as indicated the chicks used in these studies were White Plymouth Rocks from 1 to 2 days old when the experiments were begun; they were secured from stock which was blood tested for bacillary white diarrhea. The feed used was a standard chick starter mash supplied by a local milling company. The thiouracil[2] or thiourea was mixed by hand in the basal ration at several dosage levels expressed as per cent by weight. Tap water was available at all times. A 0.1 per cent thiouracil-tap water solution was prepared by the addition of the required amount of a concentrated NaOH solution. The hover temperature in the electric brooders was kept at approximately 95° F., while the room temperature varied between 78 and 82° F. The chicks kept at room temperature were housed in metal rat cages.

The chickens were killed with ether at the end of an experiment, sexed, the body weights recorded and the thyroids dissected and weighed immediately on a precision balance to the nearest 0.1 mg. All thyroid weights are reported on the basis of mg. per 100 gm. of body weight. Synthetic *d,l*-thyroxine was dissolved in distilled water with the use of NaOH, and injected subcutaneously daily in a volume of 0.1 cc.

RESULTS

Thyroid enlargement, thiouracil and thiourea in diet. Both thiouracil and thiourea were fed to groups of chicks for 14 days at the dosage levels of 0.025, 0.05, 0.1 and 0.2 per cent (fig. 1). From 14 to 27 chicks comprised a group at a given dosage level of either thiouracil or thiourea. As the two sexes were distributed in the groups at random, a given point on the figure represents results from 6 to 14 chicks. There was progressive thyroid enlargement with the use of both thiourea and thiouracil, the maximum responses being secured with both drugs at the 0.1 per cent level. There was probably no significant difference in the potency of the two drugs under these conditions, although the thiouracil was slightly superior in its action. As may be noted from the figure there was a difference in the thyroid response of the two sexes to both thiouracil and thiourea, the females giving the greater response.

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[2] The thiouracil was supplied by the Lederle Laboratories, Inc., Pearl River, New York.

Thiourea was fed to similar groups of chicks at the levels of 0.4, 0.6 and 0.8 per cent. All chicks receiving the 0.6 and 0.8 per cent dosage of the drug died, indicating that it was toxic at these levels. The group receiving 0.4 per cent reacted similarly to that receiving 0.2 per cent, both of these groups having lower thyroid weights than the group receiving 0.1 per cent.

Thus in White Plymouth Rock chicks up to 14 days of age, 0.1 per cent thiouracil or thiourea in the diet caused maximum thyroid enlargement and the thyroid glands of female chicks enlarged to a greater degree than did those of the male chicks.

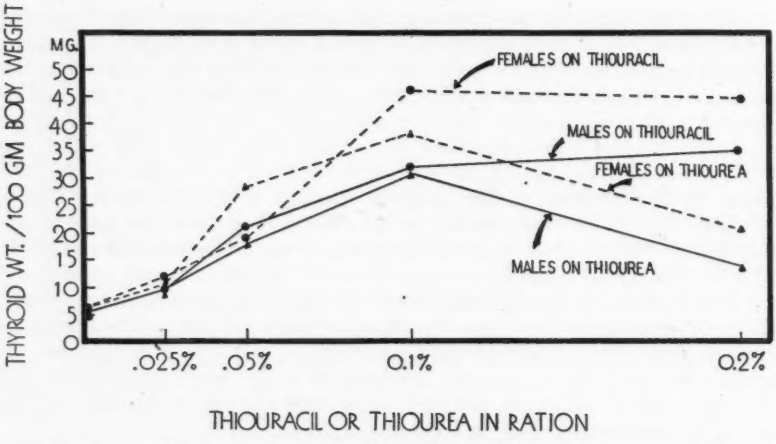

FIG. 1. Influence of increasing amounts of thiouracil or thiourea in the ration of day-old White Plymouth Rock chicks upon thyroid weight.

Time required for thyroid enlargement. Male and female chicks were kept at room temperature, fed 0.1 per cent thiouracil in the diet and groups of these were killed every second day of the experimental period up to and including 14 days. There were on the average about 8 chicks in each sex group at a given age. Smaller groups (about 5 chicks) were fed the basal ration and killed as controls (fig. 2). Thyroid enlargement in the experimental animals was first noted at 4 days and reached a maximum at 12 days of age. As shown in experiment 1, there was a marked sex difference in the thyroid reaction, the female chicks again giving the greater response throughout the course of the experiment.

Influence of thyroxine on thyroid enlargement. One-day-old chicks kept at room temperature were fed 0.1 per cent thiouracil in the diet for 14 days. Groups of these chicks received in addition to the thiouracil graded dosages of d,l-thyroxine (0.25, 0.5, 1.0, 2.0, 3.0, 4.0, 8.0, and 16.0 μg.), daily by subcutaneous injection. A normal control

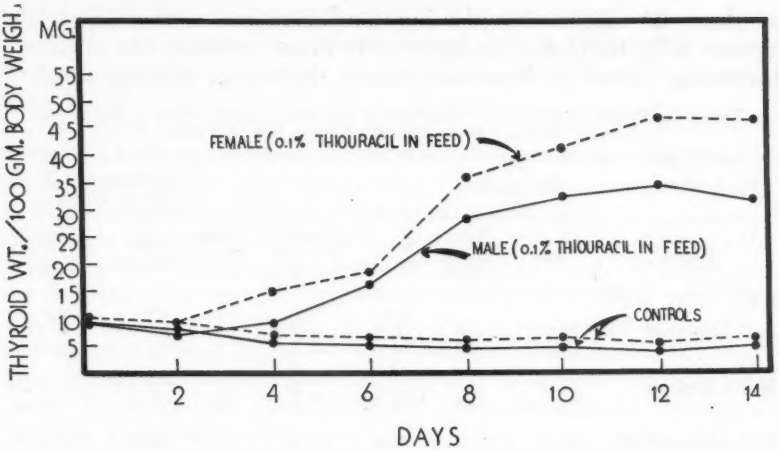

FIG. 2. Time required for the enlargement of the thyroid glands of male and female White Plymouth Rock chicks fed 0.1% thiouracil in the diet. Note greater weight of the female glands.

group and also a group receiving 0.1 per cent thiouracil only in the feed were maintained similarly. The group treated with 8.0 and 16.0 μg. of thyroxine per day had thyroid weights very similar to those receiving 4.0 μg. per day, so that the thyroid weights in the two groups treated with the highest thyroxine dosage are not included in figure 3. Each point in figure 3 was derived from data for an average

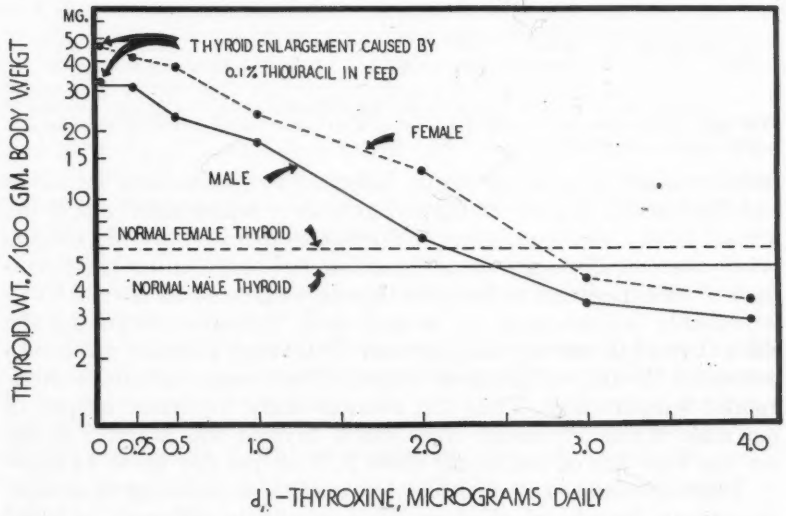

FIG. 3. Effect of increasing amounts of d,l-thyroxine upon the thyroid enlargement of White Plymouth Rock chicks fed 0.1% thiouracil in their diet.

of about 13 chicks. By plotting the logarithm of thyroid weight against daily thyroxine dosage a fairly linear response was obtained. Increasing doses of thyroxine caused decreasing thyroid weights.

TABLE 1. BREED AND SEX DIFFERENCES IN THYROID RESPONSE OF ONE-DAY-OLD CHICKS TREATED WITH THIOURACIL FOR 14 DAYS

		Controls			0.1% Thiouracil in Drinking Water		
Breed	Sex	Number of Chicks	Body Weight	Thyroid wt./100 Gm. B.W.	Number of Chicks	Body Weight	Thyroid wt./100 Gm. B.W.
			gm.	*mg.*		*gm.*	*mg.*
Buff Minorcas	Male	8	97.0	5.9	11	82.0	52.1
	Female	10	90.0	5.8	9	79.0	69.8
White Leghorn	Male	11	77.0	7.9	13	93.0	37.8
	Female	14	72.0	7.9	12	91.0	68.8
W. Leghorn-N. H. Red cross	Male	7	100.0	5.3	8	92.0	55.6
	Female	11	100.0	5.2	11	103.0	57.9
N. H. Red	Male	13	99.0	6.0	10	90.0	33.3
	Female	7	92.0	7.1	7	88.0	43.5
Buff Orpington	Male	16	101.0	5.2	7	100.0	26.9
	Female	10	95.0	5.9	9	91.0	47.0
Barred P. Rock	Male	11	76.0	5.5	12	81.0	30.1
	Female	12	64.0	6.7	12	81.0	35.4
W. P. Rock	Male	10	90.0	4.3	8	86.0	31.3*
	Female	10	92.0	4.1	10	93.0	31.4*
White Wyandotte	Male	11	102.0	5.6	12	89.0	30.2
	Female	15	88.0	6.4	13	91.0	33.4
S. C. Rhode Is. Red	Male	10	99.7	8.7	9	91.0	26.9
	Female	14	93.4	8.3	12	77.0	30.7

* 13 males kept at room temperature had thyroid glands weighing an average of 31.9 mg. and 14 females averaged 46.5 mg. This latter value probably more nearly represents the sex difference.

Between 2 and 3 μg. of thyroxine daily was needed in both the males and the females to prevent thyroid growth or enlargement above the normal level. Greater amounts of thyroxine decreased thyroid weights below normal. The amount of thyroxine necessary under the conditions of the experiment to keep the thyroid weights at the normal levels is probably indicative of the normal daily thyroxine output by the chick thyroid. Dempsey and Astwood (1943) used a similar method to determine the thyroid hormone output of rats under various environmental temperatures. Thus the average daily thyroxine output of the male White Plymouth Rock chick thyroid was about 2.45 μg. per day and that of the female chick 2.75 μg. per day up to 14 days.

Thyroid enlargement of various breeds of chicks receiving thiouracil. As various breeds of chickens have markedly different physical characteristics, it seemed interesting to determine the thyroid response to thiouracil in the different types. Three light weight breeds

were studied (S. C. White Leghorn, Buff Minorca and a Leghorn-New Hampshire cross) and 6 medium weight breeds (White Plymouth Rock, Barred Plymouth Rock, S. C. Rhode Island Red, New Hampshire, White Wyandotte and Buff Orpington).

Groups of chicks from these various breeds were kept for 14 days in electric battery brooders and were given 0.1 per cent thiouracil in the drinking water. Other groups served as controls. They were killed at the end of 14 days and the thyroid glands weighed.

As may be seen from Table 1, there is a correlation between the type of bird and the thyroid response. The Leghorns, Minorcas and the Leghorn-New Hampshire cross are examples of breeds with high egg production, a relatively light body weight and with an active nervous disposition. In these breeds the thyroid response was greater than in the heavier breeds, indicating that these birds are, perhaps, more "hyperthyroid" than the heavier breeds. Thyroid response in the females in every breed was greater than in the males. Further research will be necessary to determine if the greater thyroid response of the lighter breeds is indicative of a higher normal thyroxine output by the thyroid as compared to the heavier breeds of chickens.

DISCUSSION

It is apparent that the chick thyroid is responsive to thiouracil or thiourea when these compounds are given at an effective dosage level for 14 days to one-day-old birds. Mackenzie and Mackenzie (1943) reported no thyroid enlargement in 2-week-old chickens receiving sulfaguanidine as 2 or 3 per cent of the diet, and believed that this represented a species difference between the chicken and other species tested. Although our experiments differ from those of the Mackenzies, the difference in results can probably be explained on the basis of the dosages of thiouracil, thiourea and sulfaguanidine used.

The biological assay of materials having thyroidal activity has been very difficult and rather inaccurate up to the present time. The experiments reported in this paper could be used as the basis for a rather sensitive assay for thyroidal materials. Dempsey and Astwood (1943) presented the basis of this type of assay using the rat as the test animal. The one-day-old chick has advantages over the rat as an assay animal in its cheapness, availability and ease of handling in large numbers.

To set up a method for the assay of thyroidal materials in the chick on the basis of the inhibition of thyroid function by thiouracil or thiourea, several factors must be considered: *a*) the sex, breed and age of chick; *b*) the length of assay period; *c*) the dosage and mode of administration of the thyroid-inhibiting drug; *d*) the mode of administration of thyroidal material, and *e*) the environmental temperature.

On the basis of the experiments reported in this paper probably either sex of any of the breeds of chickens would be satisfactory for

this assay but as there is a sex difference in the response, sexed chicks would be preferred. An assay period from 10 to 14 days in length would be desirable. Thiouracil used at a 0.1 per cent level in the feed would seem to be the best drug and the best mode of administration. It would probably be preferable to administer the thyroidal material by subcutaneous injection. Baby chicks may be kept at a room temperature of 80 to 85° F., without drafts, and in ordinary rat cages with 8 to 12 chicks per cage. The thyroxine dosage curve (fig. 3), with thyroxine dosage plotted against the logarithm of thyroid weight per 100 gm. of body weight is practically a straight line response, which is very desirable for assay purposes.

The different degrees of thyroid response in the various breeds of chicks receiving thiouracil suggests the importance of determining the role of thyroxine as a contributing factor to breed differences.

SUMMARY

Approximately 0.1 per cent thiouracil and thiourea in the diet for 14 days was the optimal dosage for the production of maximal enlargement of the thyroid in one to 2-day-old White Plymouth Rock chicks. Maximum thyroid enlargement expressed as thyroid weight in mg. per 100 gm. of body weight was secured in about 12 days, when White Plymouth Rock chicks were fed 0.1 per cent thiouracil in the diet.

Female White Plymouth Rock chicks exhibited a greater thyroid response than did the males when fed 0.1 per cent thiouracil or 0.1 per cent thiourea in the feed for 14 days. Increasing dosages of thyroxine progressively decreased the thyroid enlargement produced in White Plymouth Rock chicks by 0.1 per cent thiouracil in the diet. There was a sex difference in this regard, the females requiring greater amounts of thyroxine to depress the thyroid weights to any given weight level or to the normal level for their sex.

Nine different breeds of chickens were given 0.1 per cent thiouracil in drinking water and the thyroid responses noted. The thyroids of the lighter breeds of chickens gave a greater response as compared to the thyroids of the heavier breeds. The application of these experiments in the formulation of a biological assay for materials having thyroxine-like activity was discussed.

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A MICRO METHOD FOR THE SEPARATION OF 17-KETOSTEROIDS INTO ALPHA AND BETA FRACTIONS

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LITTLE is known about the ratios of urinary alpha and beta[1] 17-ketosteroids in normal and diseased states (Baumann and Metzger, 1940; Talbot and his coworkers, 1942; Rhoads, Dobriner and their collaborators, 1942b; Conference on Ketosteroids, 1942). That such determinations may be of clinical importance is suggested by the reports that relatively large amounts of the beta form are present in cases of adrenal cortical carcinoma (Crooke and Callow, 1939; Talbot and his coworkers, 1942). Scott and Vermeulen (1942) have reported a difference in the relative amounts of alpha and beta fractions in patients with prostatic carcinoma before and after castration. The different workers have based their separation into alpha and beta ketosteroid fractions on the observation that steroids present in the beta fraction are precipitated by digitonin, but different procedures have been followed so that the results are not comparable. Of the separation methods used only two are directly applicable where relatively small amounts of 17-ketosteroid material such as may be present in single 24-hour urine specimens are available. The method employed by Talbot and his collaborators (1940a) relies on an indirect determination of the beta fraction while the procedure outlined by Baumann and Metzger (1940) is applied to urinary extracts which have been prepared according to a method not current in most laboratories.

It appeared important to develop a method in which both the alpha and beta fractions are measured directly and which can be applied for clinical purposes to short-term collections of urine. Such a method is outlined in the present paper. It may be applied in many cases to a 24-hour urine specimen, or, if the total daily 17-ketosteroid output is very small, a 2- or 3-day collection may be necessary.

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[1] The term *alpha* as used in this paper applies to the fraction which is not precipitated by digitonin and includes the non-alcoholic constituents in addition to the 3-alpha-hydroxy fraction, while the term *beta* refers to the fraction which is precipitated by digitonin under the conditions described.

METHODS

The method here described establishes standard conditions for the separation of alpha and beta 17-ketosteroids by means of digitonin precipitation of the beta steroids.

A urinary extract containing 15 mg. or more of neutral 17-ketosteroids dissolved in absolute ethanol, or a solution of pure alpha and beta 17-ketosteroids in absolute ethanol, is transferred quantitatively to a calibrated 15 cc. centrifuge tube and warmed. To it is added a warm solution of digitonin in such proportions of absolute alcohol and water that the final concentration of digitonin is 1 per cent in 90 per cent alcohol. At least 14 mg. digitonin should be allowed for each mg. of beta 17-ketosteroid expected in the test solution. For example, to 2 mg. androsterone and 2 mg. dehydroisoandrosterone in 2 cc. absolute alcohol 30 mg. digitonin in 0.3 cc. water and 0.7 cc. absolute alcohol are added. The tube is corked and allowed to stand overnight. Ten cc. pure anhydrous ether in small portions are added, stirring with a glass rod between additions, and reserving the glass rod for subsequent use. The tube is corked, centrifuged for 10 minutes at about 2,000 R.P.M., and the supernatant poured off into a 250 cc. separatory funnel. The precipitate, which contains the beta fraction, is washed 3 times with 10 cc. portions of ether, stirring and centrifuging after each addition and adding the supernatants to the separatory funnel. The combined ether extracts are washed 3 times with 25 cc. portions of water, the ether solution is drawn off into a flask, evaporated under reduced pressure and dried in a desiccator over calcium chloride. This fraction contains the material which is not precipitable by digitonin (alpha fraction).

The precipitate remaining in the centrifuge tube is dissolved in 0.5 cc. of dry pyridine and heated for 3 minutes in a hot-water bath. After cooling, 10 cc. anhydrous ether are added to precipitate the digitonin and the tube is centrifuged for 10 minutes. The supernatant is poured into a 250 cc. separatory funnel and the pyridine-ether treatment of the precipitate is repeated. The remaining precipitate is washed twice with 10 cc. ether, stirring and centrifuging each time. The combined ether-pyridine solution is washed 3 times with 10 cc. portions of 2 N sulfuric acid and 3 times with 25 cc. portions of water. The ether solution is evaporated in a flask under reduced pressure and dried in a desiccator. This fraction contains the material which is precipitable by digitonin (beta fraction).

Where the starting volume is greater than 3 cc., that is when more than 30 mg. digitonin are required to precipitate the beta fraction, it is preferable to carry out the separation in a 50 cc. centrifuge tube. The procedure here is identical with that outlined above except that 25 cc. portions of ether, 1.0 or 1.5 cc. portions of pyridine and 20 or 25 cc. portions of acid are used.

The alpha and beta 17-ketosteroids are determined quantitatively by means of the Zimmerman color reaction, using the absolute alcohol method of Callow, Callow and Emmens (1938) adapted for use with the Klett-Summerson photoelectric colorimeter.

Preparation of urine extracts. Hydrolysis and extraction of urine were performed in 2 ways. (1) The urine was simultaneously hydrolyzed and extracted, using carbon tetrachloride as the extractive, according to the method of Talbot and his collaborators (1940b). (2) Preliminary hydrolysis

by boiling for 10 minutes with 15 volumes per cent concentrated hydrochloric acid was followed by rapid cooling and extraction for 2 hours with carbon tetrachloride, using the Wolfe-Hershberg continuous extraction apparatus. The neutral fraction was prepared in the usual way (Talbot *et al.*, 1940a), and the ketonic steroids separated from the non-ketonic material by treatment with Girard's Reagent T (Girard and Sandulesco, 1936). An aliquot of the ketonic fraction was taken for a determination of 17-ketosteroids and the remaining material, or a suitable aliquot, was used for the separation of alpha and beta compounds. The amount of digitonin to be added may be determined from the assumption that except in cases of adrenal hyperplasia or adrenal tumors not more than 15 per cent of the total 17-ketosteroid content may be expected to be precipitable by digitonin.

RESULTS

Solutions of pure compounds. Table 1 presents a representative group of results obtained on solutions containing from 0.12 to 2.0 mg.

TABLE 1. RECOVERY OF PURE ANDROSTERONE AND DEHYDROISOANDROSTERONE

Added		Volume*	Recovered			
And.	DHA		And.	DHA	And.	DHA
mg.	*mg.*	*cc.*	*mg.*	*mg.*	%	%
	0.12	3		0.118		98
	0.12	3		0.125		104
1.0	0.12	3	1.02	0.118	102	98
	0.25	2		0.245		98
	0.50	2		0.494		99
	0.50	10		0.465		93
	1.0	3		1.01		101
	1.0	10		1.02		102
	2.0	10		1.92		96
2.0	2.0	3	1.97	1.94	99	97

* Refers to volume after addition of digitonin.

dehydroisoandrosterone and on mixtures containing 1.0 to 2.0 mg. androsterone and 0.12 to 2.0 mg. dehydroisoandrosterone.[2] The percentage recoveries ranged from 93 to 105 per cent, and from a total of 18 experiments on dehydroisoandrosterone alone the average recovery was 99 per cent, while from a total of 10 experiments on mixtures of androsterone and dehydroisoandrosterone the average recoveries were 99 and 97 per cent respectively. The recoveries are not dependent on the initial volume of the ketosteroid-digitonin solution since volumes of 2, 3 or 10 cc. were used in different experiments, nor are they interfered with by an excess of digitonin.

Recovery of dehydroisoandrosterone from urine extracts. The results of experiments on the recovery of pure dehydroisoandrosterone from neutral ketonic urinary extracts are given in Table 2. It will be noted that when amounts of dehydroisoandrosterone ranging from 0.25 to

[2] The author is indebted to Ciba Pharmaceutical Products, Inc., for a supply of the crystalline androsterone and dehydroisoandrosterone. The former has a melting point of 182° and the latter of 148°.

1.0 mg. are added the experimental recoveries differ from the calculated recoveries by 0.05 to 0.12 mg. There is a tendency for the recoveries to be high where the larger amounts of sterone were added, but the discrepancies are slight and become almost negligible when the beta fraction is expressed in terms of percentage of total 17-ketosteroid content. The results of the 2 determinations of the alpha fraction for each extract are in satisfactory agreement.

TABLE 2. RECOVERY OF DEHYDROISOANDROSTERONE ADDED TO NEUTRAL KETONIC URINARY EXTRACTS

Extract No.	Total 17-KS mg.	DHA added mg.	Alpha mg.	Beta mg.	Recovery mg.	Recovery %
1	9.3		8.45	0.37		
	9.3	0.25	8.40	0.57	0.20	80
2	10.6		10.0	0.16		
	10.6	0.25	10.0	0.41	0.25	100
3	19.0		17.8	0.82		
	19.0	0.50	17.5	1.40	0.58	116
4	22.2		21.7	0.90		
	22.2	0.50	21.6	1.52	0.62	124
5	10.8		9.5	0.43		
	10.8	1.0	9.5	1.53	1.10	110
6	17.7		16.2	0.57		
	17.7	1.0	15.7	1.67	1.10	110

Urinary extracts. Table 3 presents a preliminary report on the application of the method to neutral ketonic urinary extracts. Quantitative determinations were made on both the digitonin-precipitable and the digitonin-non-precipitable fractions, and the total recoveries of the two fractions varied from 85 to 100 per cent of the total ketonic assays.

A comparison of the results with the two methods of hydrolysis and extraction of 12 urine samples showed that the method using preliminary hydrolysis and subsequent extraction gave results for the total neutral ketonic fractions 25 to 45 per cent higher than did the simultaneous hydrolysis and extraction procedure. Of the results in Table 3 cases (1), (2), (4) and (11) were simultaneously hydrolyzed and extracted whereas the remaining cases were subjected to preliminary hydrolysis. Separation into alpha and beta fractions was carried out on 3 of the 12 samples subjected to both methods of hydrolysis and extraction. In all 3 cases the total amounts of beta material were in fairly close agreement with the 2 procedures, being slightly higher where preliminary hydrolysis was performed. The discrepancy in results with the 2 methods was accounted for almost entirely in the digitonin-non-precipitable fraction where the method

of simultaneous hydrolysis and extraction yielded significantly smaller amounts.

DISCUSSION

A micro method has been described for the partition of 17-ketosteroids into alpha and beta fractions based on the modification by Dobriner *et al.* (1942a) of the procedure of Butler and Marrian (1938). These investigators have applied the method to long-term collections of urine whereas the present modification is applicable to small

TABLE 3. FRACTIONATION OF URINARY 17-KETOSTEROIDS

Subject	Sex	Age	Diagnosis	Urine Collection hrs.	Total mg./24 hrs.	Alpha mg./24 hrs.	Beta mg./24 hrs.	Beta %
(1) V.H.	F	24	Normal	72	7.0*	6.6	0.11	1.6
(2) M.C.	F	23	Normal	72	6.9*	6.2	0.06	0.9
(3) M.T.	F	42	Normal	72	7.0*	6.5	0.15	2.1
(4) D.G.	F	49	Normal	72	9.7*	8.2	0.23	2.4
(5) F.B.	M	52	Normal	24	13.8	12.5	0.56	4.1
(6) J.C.	M	62	Normal	72	6.1*	5.9	0.09	1.5
(7) D.L.	F	20	Adrenal Hyperplasia	24	43.9	39.3	4.4	10.0
(8) M.S.	F	9	Adrenal Hyperplasia	24	51.6	46.7	4.4	8.5
(9) J.S.	F	11	Adrenal Hyperplasia	24	56.5	48.9	7.1	12.6
(10) D.C.	F	20	Adrenal Hyperplasia	24	48.8	43.0	3.4	7.0
(11) R.L.	M	69	Prostatic Carcinoma	72	3.0*	2.5	0.24	8.0
(12) G.G.	M	68	Prostatic Carcinoma	72	5.0*	4.3	0.08	1.6

* Average of 72-hour collection.

amounts of material. As has been shown in Table 1 dehydroisoandrosterone, which appears to be the main component of the digitonin-precipitable fraction of urinary neutral 17-ketosteroids, can be recovered satisfactorily when amounts as small as 0.12 mg. are present. The method is best applied to urinary extracts which contain 15 mg. or more of 17-ketosteroids. Such an amount is present in some cases in a 24-hour urine collection, while in other cases a 48- or 72-hour collection may be necessary.

The results of the application of the method to urinary extracts, as presented in Table 3, are in general agreement with those obtained by Baumann and Metzger (1940). These workers did not apply a Girard separation of the ketonic material before partition into alpha and beta fractions but used the method of magnesium oxide adsorption to eliminate interfering chromogenic material. The values in Table 2 are in the same range, also, as those reported by Rhoads, Dobriner *et al.* (1942b). On the other hand, the values for the beta fraction are considerably lower than those obtained by Talbot and

his coworkers (1942), particularly for normal individuals. The method used by the latter investigators depends on an indirect determination of the beta fraction, obtained as the difference between the value of the total neutral fraction and that of the fraction not precipitated by digitonin. As may be seen from Table 3, if such a procedure were followed in the present method relatively higher values for the beta fraction would be obtained in some cases. The discrepancy can be explained as being due to the elimination of chromogenic non-ketonic material during the alpha-beta separation procedure.

SUMMARY

A micro procedure has been described for the separation of urinary 17-ketosteroids into alpha and beta components, such determinations having been reported to be of clinical significance. The value of the present method lies in its applicability to short-term urine collections (1- to 3-day), and in its direct measurements of both alpha and beta fractions with a consequent avoidance of the increased experimental error inherent in a procedure where the beta fraction is measured by difference.

In experiments on solutions of androsterone (an alpha compound) and of dehydroisoandrosterone (a beta compound), 94 to 102 per cent of the former was recovered when 1.0 to 2.0 mg. were present, and 93 to 105 per cent of the latter was recovered when 0.12 to 2.0 mg. were present. A preliminary report is given on the application of the method to urinary extracts from normal and pathological subjects.

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THE EFFECTS OF ADRENALECTOMY ON THE ACTIVITY OF CYTOCHROME OXIDASE AND THE CONCENTRATION OF CYTOCHROME C IN RATS[1]

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ADRENALECTOMY results in disturbances in the respiratory metabolism of various tissues according to the reports of several investigators. Himwich and his collaborators (1934) found that the Q_{O_2} of testicular tissue and of minced cerebral tissue decreased after adrenalectomy in rats. There was no significant depression in the respiration of liver slices. Crismon and Field (1940) also working with rats reported that the respiration of kidney slices decreased but found no change in skeletal muscle or brain. Tipton (1941) reported a decreased Q_{O_2} in liver slices and also in kidney slices removed from rats 7–10 days after adrenalectomy. A decrease in the respiration of kidney slices was found also by Russell and Wilhelmi (1941a). The decrease in Q_{O_2} was less when the adrenalectomized rats were treated with salt. Treatment with adrenal cortical extract and with desoxycorticosterone restored the kidney respiration to normal values. Koepf, *et al.* (1941), on the contrary, found that the respiration of liver slices in their rats was unaltered after adrenalectomy. However, their values for liver Q_{O_2} from normal animals are considerably lower than those which are usually reported.

It is well known that deficiencies of factors of the adrenal cortex are followed by disturbances in the metabolism of carbohydrate. Long (1942) discussed the various metabolic reactions that may be affected by decreased cortical hormone. Russell and Wilhelmi (1941a, 1941b) found a reduced capacity in kidney tissue for carbohydrate formation from amino acids which Koepf and his collaborators (1941) did not confirm on liver slices from adrenalectomized rats. However the latter investigators reported a reduced capacity for carbohydrate formation from intermediary substances such as pyruvate.

Our earlier report (1941) that liver slices from adrenalectomized rats showed a slower rate of respiration in the presence of sodium

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succinate and sodium pyruvate than did those from normal animals suggests that there may be hormonal regulation of some or all of the respiratory enzymes involved in the oxidation of these substances. Cytochrome oxidase and its substrate cytochrome *c* are common to both systems and the present study reports a decrease in these substances in liver, kidney, and heart tissue after adrenalectomy.

MATERIALS AND METHODS

Young male rats weighing from 80 to 120 grams were divided into five groups. Rats from the strain used in this study usually die about ten days after adrenalectomy if untreated. All animals were given 1% NaCl as drinking water until the 3rd day after the operation when they were changed to ordinary tap water, unless the effect of maintenance on salt solution was to be studied. The first group of rats were controls; some of them were subjected to sham operations but since no significant difference was observed they are placed in Group 1 along with the other controls. Group 2 was composed of unoperated rats fed the same amount of Purina dog chow that the adrenalectomized rats consumed; thus they were partially starved for almost a week. Group 3 animals were adrenalectomized, then divided into two subgroups, an *a* group sacrificed for study 6 days after operation, and a *b* group sacrificed 10 to 12 days after the operation when they were approaching a condition of crisis. Group 4 were adrenalectomized animals which were given 1% NaCl solution to drink for a week after operation until they were sacrified for the experiment. Group 5 was made up of rats adrenalectomized and treated with adrenal cortical extract (Eschatin).

For the determination of the activity of cytochrome oxidase and the concentration of cytochrome *c* the rats were decapitated and bled. After the bleeding the liver, kidneys, and heart were removed and weighed after rinsing in 0.025 M phosphate buffer solution (pH 7.2). An aliquot was taken for homogenization in 30 volumes of M/15 dibasic potassium phosphate. The remainder of the tissue or a second aliquot was used to determine water content so that the total dry weight of the organ could be determined. One cubic centimeter of the homogenate was used for each test. The manometric method of Stotz was used (1939) as modified by Schultze (1939). The cytochrome *c* was prepared from beef heart by the method of Keilin and Hartree (1937) and analyzed for iron by Drabkin's method (1941). The equilibration was in air at a bath temperature of 38° C. Boiled tissue homogenate as suggested by Stotz (1939) was used as a control. The oxygen uptake per hour in the presence of excess cytochrome *c* was almost exactly proportional to the dry weight of the tissue homogenate used, at least up to 3 mgms. dry weight of sample. The method then is a reliable measure of cytochrome oxidase activity.

RESULTS

The results obtained on the various groups of rats are presented in Table 1. The cytochrome oxidase activity although varying considerably from animal to animal is greatest in heart tissue, next in kidney, and lowest in the liver tissue. This order of activity agrees with that previously reported by Stotz (1939). The cytochrome oxi-

dase activities found in our experiments fall between those of Schultze (1939) and those of Axelrod, Swingle, and Elvehjem (1942). Tissues from adult rats appear to have somewhat lower values than those from young ones, a finding reported previously by Axelrod and his collaborators, but our young rats have not shown Q_{O_2} values for cytochrome oxidase in liver tissue as high as the 500 to 600 that these authors published. The cytochrome *c* concentration appears to parallel the cytochrome oxidase so far as the order of activity from tissue to tissue is concerned, being highest in the heart tissue, next in kidney, and lowest in the liver, which has also been previously reported by the above mentioned authors.

TABLE 1. CYTOCHROME OXIDASE Q_{O_2} AND CYTOCHROME *C* CONCENTRATION OF HEART, KIDNEY, AND LIVER TISSUE OF RATS UNDER VARYING CONDITIONS*

The figures are average Q_{O_2} for the group. The figures in parentheses show the range of variation

Group No.	No. of Animals	Cytochrome Oxidase			Cytochrome *c*		
		Heart	Kidney	Liver	Heart	Kidney	Liver
		Q_{O_2}	Q_{O_2}	Q_{O_2}	*μg./mg. dry wt.*	*μg./mg. dry wt.*	*μg./mg. dry wt.*
1	30	694 (674–724)	570 (544–624)	300 (274–324)	3.0 ± .2	1.5 ± .15	0.4 ± .1
2	12	760 (735–810)	660 (645–705)	375 (350–405)			
3a	20	610 (570–625)	501 (475–515)	250 (210–265)	2.87 ± .17	1.0 ± .15	0.3 ± .04
3b	16	585 (550–595)	485 (450–510)	200 (190–220)	3.02 ± .2	0.9 ± .1	0.32 ± .05
4	20	630 (600–665)	515 (480–525)	260 (215–275)			
5	6	675 (670–682)	555 (530–580)	278 (260–300)			

* Group 1, normal rats fed; Group 2, normal rats on restricted food intake; Group 3a, adrenalectomized, untreated rats, 6 days postoperative; Group 3b, adrenalectomized, untreated, 10–12 days postoperative; Group 4, adrenalectomized rats given 1% NaCl for drinking water; Group 5, adrenalectomized rats treated with adrenal extract.

The rats in Group 3, adrenalectomized with no subsequent treatment, show a significant decrease in cytochrome oxidase activity of all three tissues studied, and a decrease in cytochrome *c* concentration in the kidney and liver tissues. The rats in Group 2 of Table 2 show that undernutrition produces a rise in cytochrome oxidase Q_{O_2}. However, the total cytochrome oxidase of the kidney and the liver of these rats remains about the same as that of normal rats (Group 1 rats, unoperated fed *ad lib.*), which indicates that undernutrition in our experiments had little effect on the total cytochrome oxidase activity.

Both the liver and kidney make up a smaller percentage of the total body weight in Group 2 than in the normal rats of Group 1. In the adrenalectomized groups the percentage body weight decreases still more, but the cytochrome oxidase Q_{O_2} of the total liver (c.mm.

per hr. for the total dry weight of the organ) is significantly lower and the Q_{O_2} as mentioned above is lower. Administration of one per cent sodium chloride solution as drinking water prevented some of the decrease in oxidase activity, while administration of adrenal cortical extract prevented almost entirely the decrease in the oxidase activity. Group 3a differs very little from 3b as is shown in Table 1. The cytochrome oxidase activity does not change very much as the animal approaches crisis. The difference between Group 3a and 3b may easily be due to the moribund condition which is present or developing.

Values obtained by multiplying the Q_{O_2} by the total dry weight of the liver have been taken as a measure of the cytochrome oxidase

TABLE 2. PER CENT BODY WEIGHT AND TOTAL CYTOCHROME OXIDASE ACTIVITY IN KIDNEY AND LIVER OF 100 GRAM RATS UNDER CONDITIONS OF TABLE 1

Group No.	Kidney		Liver	
	Per cent body weight	*Total cytochrome oxidase* $Q_{O_2} \times$ *total dry weight*	*Per cent body weight*	*Total cytochrome oxidase* $Q_{O_2} \times$ *total dry weight*
1	0.47 ± .013	60,277.5 ± 4200	4.48 ± .22	381,000 ± 15,000
2	0.42 ± .03	58,300.0 ± 5500	4.01 ± .20	390,000 ± 10,000
3a	0.39 ± .015	39,078.0 ± 2500	3.0 ± .25	239,000 ± 22,000
4	0.41 ± .02	42,156.0 ± 3000	3.5 ± .15	300,000 ± 17,000
5	0.49 ± .013	61,500.0 ± 4500	4.6 ± .2	375,000 ± 15,500

activity of the entire liver (Axelrod, Swingle, and Elvehjem 1942). The average per cent body weight and the total cytochrome oxidase Q_{O_2} for liver and kidney for an animal weight of 100 grams is shown in Table 2. Groups 1 and 2 show about the same total cytochrome oxidase although Group 2 shows an appreciably higher Q_{O_2} due to the loss in dry weight of the liver and kidney during partial starvation. There is a fall in the total cytochrome oxidase in the adrenalectomized animals which is prevented partly by salt administration and completely by cortical extract.

DISCUSSION

There appears to be a significant decrease in the cytochrome oxidase Q_{O_2} of heart, liver, and kidney tissue at the same time after adrenalectomy as one finds decreased tissue Q_{O_2} and a decreased respiratory response to excess succinate and pyruvate added to the isolated tissue slices. The decrease in the oxidase may well have some part in the total decrease since experiments with inhibitors specific for the oxidase have shown that the bulk of the tissue respiration proceeds by way of the cytochrome oxidase, and it is an essential part of the succinate and pyruvate metabolizing systems. In the untreated adrenalectomized rats, however, there is still a high oxidase activity as shown in Table 1 and the total oxidase activity of the or-

gan is still very strong as the data in Table 2 show. If cytochrome oxidase and cytochrome *c* are present in excess, as might easily be possible in a system of such importance—a system which at times may have to deal with greatly increased metabolic demands—a fall in oxidase Q_{O_2} in liver from 300 to 250 (Table 1) shown by the untreated adrenalectomized rat may not be very significant so far as the total respiratory metabolism of the tissue is concerned. It does suggest that a deficiency of the adrenal cortical hormones does not exert any specific effect on the activity of the cytochrome oxidase system. The activity of the oxidase is still quite high just before the animal succumbs due to insufficiency as the data on the Group 3 rats show (Table 1). The experimental data reported by Schultze (1939) show that the cytochrome oxidase activity can be affected markedly by dietary deficiencies. The anorexia and poor intestinal absorption of the adrenalectomized rat make it difficult to control the type and quantity of the diet. The poor nutrition may have some action on the oxidase, although the same effect is not observed in rats of Group 2 in Table 1. It would be interesting to study the effects of vitamin supplements. Adrenalectomy and poor nutrition decrease the liver weight-body weight ratios but the latter has no appreciable effect upon the total oxidase activity (Table 2).

The decrease in cytochrome oxidase activity after adrenalectomy is all the more pronounced when it is compared with the decrease in normal animals on restricted food intake. The administration of salt maintains a better appetite in operated rats but adrenal extract is necessary to maintain a normal food consumption in such animals. Since both salt and extract treatments maintain the food intake, the adrenal extract being much more effective, treated adrenalectomized rats should be compared with Group 1 normal animals in Table 1. It is seen that salt administration prevented some of the decrease in cytochrome oxidase Q_{O_2}, whereas adrenal extract prevented the decrease almost completely. The data in Table 2 show that adrenal extract maintained the total cytochrome oxidase activity of kidney and liver of adrenalectomized rats in the range found in normal animals on unrestricted food intake.

The difference in the oxidase activity between heart, kidney, and liver tissues is considerably less in our experiments than that reported by Stotz (1939). As is shown in Table 1 heart tissue has an oxidase approximately twice as active as that of liver. In Groups 3a and 3b, the untreated adrenalectomized rats, the difference is greater but still much less than the 500 per cent difference found by Stotz.

The strong activity of the oxidase persisting in the severely insufficient animal makes it important to study the possible disturbances in other components of the tissue respiratory system such as the flavoproteins and the pyridinproteins.

SUMMARY

The activity of cytochrome oxidase and the concentration of cytochrome *c* in heart, kidney, and liver tissues were studied by chemical methods of determination in normal and adrenalectomized rats. Some of the adrenalectomized rats were treated with adrenal cortical extract, others given sodium chloride in their drinking water.

In addition to these groups one group of normal rats was studied after partial starvation.

The untreated adrenalectomized rats show a significant decrease in cytochrome oxidase activity in all tissues studied and a decrease in the concentration of cytochrome *c* in kidney and liver tissue. Treatment with sodium chloride prevented some of the decrease while adrenal extract (Eschatin) was much more effective, preventing the decrease almost completely. The partially starved group of normal animals showed a somewhat higher cytochrome oxidase Q_{0_2} than fed normals, but there was no significant change in the total liver oxidase and kidney oxidase (Q_{0_2} multiplied by the total dry weight of the organ) of this group. The total liver oxidase and kidney oxidase of the untreated adrenalectomized animals showed a decrease from the level of the normal rats.

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THE EFFECT OF PREVENTING ACAPNIA ON ADRENAL CORTICAL HYPERTROPHY UNDER CONDITIONS OF DECREASED BAROMETRIC PRESSURE

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NEW ORLEANS, LOUISIANA

Several investigators have reported that when animals are subjected to reduced barometric pressure hypertrophy of the adrenal cortex results (Armstrong and Heim, 1938; Dohan, 1942; Thorn, *et al.* 1942; Langley and Clarke, 1942). Langley and Clarke suggested that a possible cause for the stimulation of the adrenal cortex was the alkaline shift caused by the hyperventilation in response to anoxia.

To test this hypothesis it was decided to subject rats to reduced barometric pressure in the presence of sufficient carbon dioxide to prevent any acapnia and hence any alkaline shift from developing. It was expected, that, if the above assumption were correct, under these conditions no hypertrophy would occur.

METHODS

Thirty-three male albino rats of the Wistar strain weighing between 125 and 150 gm. were divided into three groups. Group A (10 animals) was used as a control group and without receiving any special treatment the animals were killed, and the adrenals removed and weighed. Group B (11 animals) was subjected to a reduced barometric pressure of 300 mm. Hg in the presence of air for 7.5 hours per day for 5 days and group C (12 animals) was subjected to a barometric pressue of 280 mm. Hg in the presence of 15 per cent carbon dioxide and 18 per cent oxygen also for 7.5 hours per day for 5 days. At the end of the fifth day the animals of groups B and C were killed and the adrenals removed and weighed.

RESULTS

The results are summarized in figure 1. The weights of the adrenal glands of group B subjected to a barometric pressure of 300 mm. Hg in the presence of air averaged 32.73 mg. varying between 28 mg. and 39.6 mg. This represents a hypertrophy of 29 per cent since in the untreated group (A) the average adrenal weight was 25.7 mg. with variations between 29.8 mg. and 21 mg. The adrenal glands of those rats subjected to 280 mm. Hg in the presence of 15 per cent CO_2

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and 18 per cent O_2 (group C) averaged 26.94 mg. with a range of 23.6 mg. to 31.9 mg. The weights of the adrenal glands of the latter group are not believed to be significantly different from those of the control group. It is important to note that the lowest value in the group showing hypertrophy is above the average of either the control group or the group subjected to reduced pressure in the presence of CO_2.

DISCUSSION

These results demonstrate the fact that preventing acapnia at high altitude will prevent adrenal cortical hypertrophy. This result could be explained in one of several ways: 1) by alleviating the anoxia,

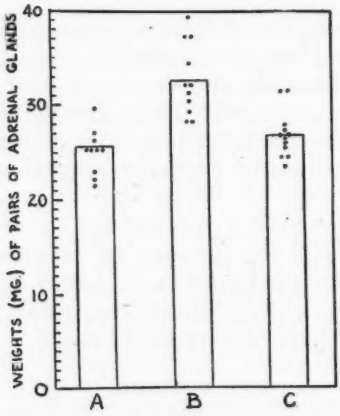

FIG. 1. The Effect of Preventing Acapnia on Adrenal Cortical Hypertrophy under Conditions of Decreased Barometric Pressure. A, control group of 10 rats; B, group of 11 rats subjected to 300 mm. Hg in the presence of air; C, group of 12 rats subjected to 280 mm. Hg in the presence of 15 per cent CO_2 and 18 per cent O_2.

2) by preventing a disturbance in the acid-base balance, or 3) by preventing a disturbance in carbohydrate metabolism. (It is known that both carbon dioxide (Evans, 1942) and the adrenal cortex are involved in the metabolism of carbohydrates.)

The presence of carbon dioxide greatly offsets the effect of anoxia. It does this by 1) increasing the respiratory minute volume and thus raising the O_2 tension in the lungs, 2) increasing the ease with which hemoglobin gives up O_2 to the tissues, 3) increasing the venous return, 4) increasing the efficiency of the carotid sinus mechanism, and 5) causing a dilatation of cerebral vessels with increased cerebral blood flow. To rule out the factor of the carbon dioxide preventing the adrenal hypertrophy by offsetting anoxia, the experiments in which carbon dioxide was used were run under conditions in which the partial pressure of oxygen was 50 mm. Hg (18 per cent of 280 mm. Hg). The partial pressure of CO_2 was 42 mm. Hg (15 per cent of 280 mm.

Hg). As stated above no hypertrophy resulted under these conditions. In the group in which the 29 per cent hypertrophy occurred the partial pressure of O_2 was 63 mm. Hg (21 per cent of 300 mm. Hg), 13 mm. more than the group in which CO_2 was used. It is difficult to say whether the presence of CO_2 can more than offset a reduction of 13 mm. Hg in the partial pressure of oxygen but in a recent report it was not able to offset a reduction of 14.3 mm. Hg (Johnson, Eckman, Rumsey, and Barach, 1942). Therefore, it is believed that the presence of carbon dioxide did not prevent adrenal hypertrophy by decreasing the anoxia but by some other mechanism. Another fact strengthening this belief is that when additional experiments were carried out reducing the pressure to 260 mm. Hg in the presence of 15 per cent CO_2 and 18 per cent oxygen, most of the animals died indicating that at 280 mm. Hg (18 per cent O_2; 15 per cent CO_2) the O_2 partial pressure was near the fatal level.

It is suggested that the stimulus for adrenal hypertrophy under conditions of decreased barometric pressure is a disturbance in the normal balance of basic cations and acidic anions. At high altitude there is a decrease in the acidic anions resulting from a decrease in the bicarbonate fraction. This leaves a relative excess of basic cations which must be eliminated to restore the balance. Since sodium makes up the greater part of the plasma base it is the one to be excreted. The relative excess of base stimulates the adrenal cortex either directly or indirectly through the pituitary to liberate additional amounts of a hormone which causes the excretion of sodium in the urine thus restoring the balance. (It has been shown that the carbohydrate regulating hormone of the adrenal cortex causes an *increased* output of sodium in the urine and that the urinary sodium is increased under conditions of decreased barometric pressure in normal animals while it is unchanged in adrenalectomized animals (Lewis, Thorn, Koepf and Dorrance, 1942).) Hence, when the acapnia is prevented there is no disturbance in the acid-base balance and no stimulation of the adrenal cortex.

It is interesting to observe that preventing the acapnia also prevents the increase in liver glycogen brought about by the adrenal cortex under conditions of decreased barometric pressure (Lewis, Thorn, Koepf and Dorrance, 1942; Nims, 1942). This finding can be explained by the fact that if there is no disturbance in the acid-base balance there is no stimulation of the adrenal cortex and hence there is no increase in the output of the hormone which causes increased sodium excretion as well as glyconeogenesis. In addition, the depletion of lipoid in the adrenal cortex due to anoxia is significantly less if the partial pressure of CO_2 is kept above about 20 mm. Hg (Langley, 1942).

As mentioned above carbon dioxide does have a direct effect on carbohydrate metabolism which in turn may influence the adrenal

cortex. However, in the light of present evidence it seems more likely that the primary effect of acapnia is on the acid-base balance.

SUMMARY AND CONCLUSIONS

A group of albino male rats subjected to 280 mm. Hg in the presence of 15 per cent CO_2 and 18 per cent O_2 for 7.5 hours per day for 5 days did not develop adrenal hypertrophy whereas a similar group subjected to 300 mm. Hg for the same length of time in the presence of air showed a 29 per cent hypertrophy. Evidence is given which tends to show that the presence of CO_2 did not act by offsetting the anoxia but rather by preventing a disturbance in the acid-base balance.

It is suggested that the adrenal cortex is stimulated by either a relative or absolute increase in basic cations or acidic anions. In the former case the adrenal cortex secretes a hormone increasing the excretion of sodium in the urine and in the latter case a hormone preventing the excretion of sodium in the urine.

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THE QUANTITATIVE ASSAY OF ADRENAL CORTICAL HORMONES BY THE MUSCLE-WORK TEST IN THE ADRENALECTOMIZED-NEPHRECTOMIZED RAT

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In 1931 the author began studies of the ability of the rat to sustain muscular responsiveness to faradic stimulation. Heron, Hales, and Ingle (1934) found that under suitable experimental conditions a high rate of work output could be sustained for several days even when no food was administered. We were impressed by the possible usefulness of work performance as an index of normality since the conditions of the experiments represented a severe stress under which the animal had to utilize endogeneous sources of energy. Ingle (1933) and Hales, Haslerud and Ingle (1935) found that muscular responsiveness was lost within a few hours following removal of the adrenal glands but that the ability to continue work was maintained by the administration of potent extracts of beef adrenal glands. Although the maintenance of life is the most important objective to be attained in treating adrenal cortical insufficiency and is the most important criterion to be used in judging the efficacy of cortical extracts and cortical steroids, it was recognized that the maintenance of life under conditions which are optimal for survival does not fully test the effectiveness of the replacement therapy. It was therefore, suggested that substitution therapy, should also be evaluated in terms of its ability to maintain a normal level of energy output in adrenalectomized animals under conditions of severe stress. The muscle work test provided such a criterion. The procedure was adopted (Ingle, 1936) as a semi-quantitative method for the assay of adrenal extracts and compounds prepared in the laboratory of Dr. E. C. Kendall at the Mayo Clinic and was so used during 1934 to 1938. These studies were continued at the George S. Cox Medical Research Institute, University of Pennsylvania during 1938 to 1941. During this time no attempt was made to standardize the work test as a procedure for quantitative assay of adrenal cortical hormones or to recommend its adoption for use by others. It was designed to meet the requirements of special research projects and the need for more simple assay procedures was recognized. However, a great deal of evidence supporting the useful-

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ness of the method has accumulated during the past ten years. The original considerations that this method was rapid, quantitative, objective, consistent and required only small amounts of the test substances can be extended as follows. The method now has a history of usefulness in guiding the chemist during fractionation of adrenal cortical extracts. It was by this method that biologic activity was first demonstrated in an adrenal steroid (Mason, Myers and Kendall, 1936). It was by this method (Ingle, 1939) and by the glycotropic test applied independently by Long and coworkers (Long, Katzin and Fry, 1940) that qualitative differences between the biologic activities of 11-desoxycorticosterone and the 11-oxygenated compounds of the adrenal cortex were first demonstrated. The method has proven to be highly specific for identifying those adrenal steroids which effect the metabolism of carbohydrate. A very large number of fractions of adrenal cortical extracts, adrenal steroids and chemically related compounds have been examined for the presence or absence of the biologic activity characteristic of the 11-oxygenated adrenal steroids without any known error.

An investigation of the experimental conditions which influence the usefulness of the work performance test as an assay method has been carried out in this laboratory. It was found that the adrenalectomized-nephrectomized rat is a satisfactory test animal. The experimental conditions have been standardized, the sensitivity of the test studied and a unit of activity defined. This paper is concerned with a description of the apparatus and the method and with data illustrating a sensitivity and consistency of the method.

APPARATUS AND METHODS

The apparatus used to stimulate the muscle was a modification of that described by Heron, Hales and Ingle (1934). A diagram of the circuit is shown in figure 1. A direct current which was rectified from the house light AC circuit was the source of the principal component of the shock. In addition, a small amount of AC current was introduced into the output of the stimulator. The intensity of both the AC and the DC components of the stimulus were controlled by means of variable resistors. The frequency of stimulation was controlled by a contactor driven by a small telechron motor which closed the circuit for a period of approximately 25 sigma three times each second. The contraction of the muscle was induced by a summation of the make and break stimuli of the current. A diagram of an oscillographic record of the shock is included in figure 1.

The contractions of the muscle were transmitted to the pulley of an automatic device which recorded the distance the muscle lifted a weight at each contraction. This recorder was a one-way direct recording Veeder counter to which was attached a compact clutch built within the pulley of the counter. A diagram of the construction of the clutch is shown in figure 2. The records of work were read from the dial of the counter and expressed as the number of recorder revolutions per unit time. Each recorder revolution represents a distance of approximately 4 cm. of muscle shortening. Since the muscle was

weighted with 100 grams each recorder revolution represented approximately 400 gram centimeters of work. The amount of work required to overcome friction and inertia was essentially a constant and was not considered in the calculations. The relative error among work recorders which are properly built and adjusted does not exceed 10 per cent.

The animals were mounted on a board which accommodates six rats. The distal end of the left tibia of the rat was exposed and secured in a vertical position by a hemostat (Halstead, 5 inch, straight, mosquito forcep). A silver

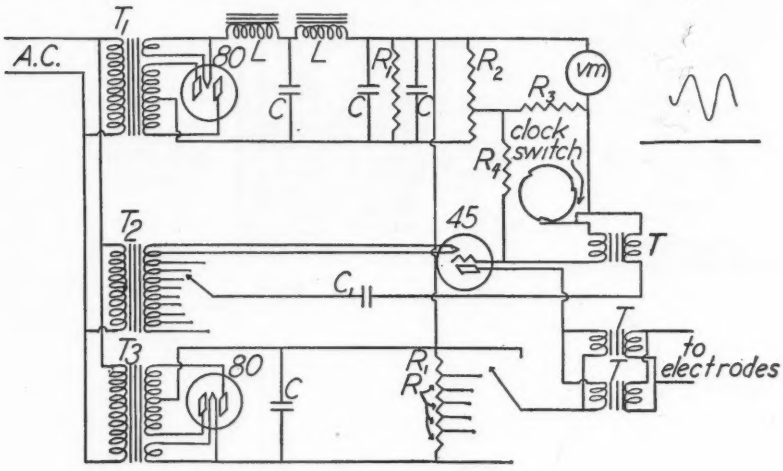

FIG. 1. Diagram of Muscle Stimulator Circuit.

R —2500Ω
R_1—10,000Ω
R_2—500,000Ω
R_3—150,000Ω
R_4—1.0 megohm
C —8 fd 600 volt
C_1—0.5 fd 600 volt
T —Thordarson T-2999 audio transformer, Ratio 3:1, 10,000 ohm primary
T_1—RCA Transformer—approximately 250 volts, DC output
T_2—Multitap transformer with taps at 0.4, 0.8, 2.5, 3.75, 5.5, 13.6, 25.0 and 30.0 volts
T_3—Thordarson T-13R05—700 volts @ 70 M.A.C.T.
L —30 henry choke
clock switch—notched wheel driven by telechron clock movement. The switch gives an impulse of approximately 25σ duration.
Oscillographic record of shock is shown at the right.

needle was inserted between the gastrocnemius and biceps muscles exactly on a horizontal level with the knee joint. The hemostat and needle served as electrodes. A 100 gram weight was attached by a strong linen thread to the freed tendon of the gastrocnemius muscle. The thread was looped around the pulley of the recorder. The electrodes of all of the animals were connected in series. A switch was provided for each animal so that it could be shorted out of the circuit when desired. Figure 3, is a diagram showing the manner of mounting the animal on the board and the positions of the electrodes and

recorder. The animal board was enclosed within a cabinet having a thermostatic control over the heating unit which consisted of six heater bulbs. Following the beginning of stimulation the temperature of the cabinet was maintained at 28 degrees C. The pre-operative maintenance of the animals and the experiments on assay were carried out in a room where both temperature and humidity were kept within narrow limits. The maintenance of a constant temperature is essential to the success of this method of assay. It has been shown that a change in temperature of as little as one degree C.

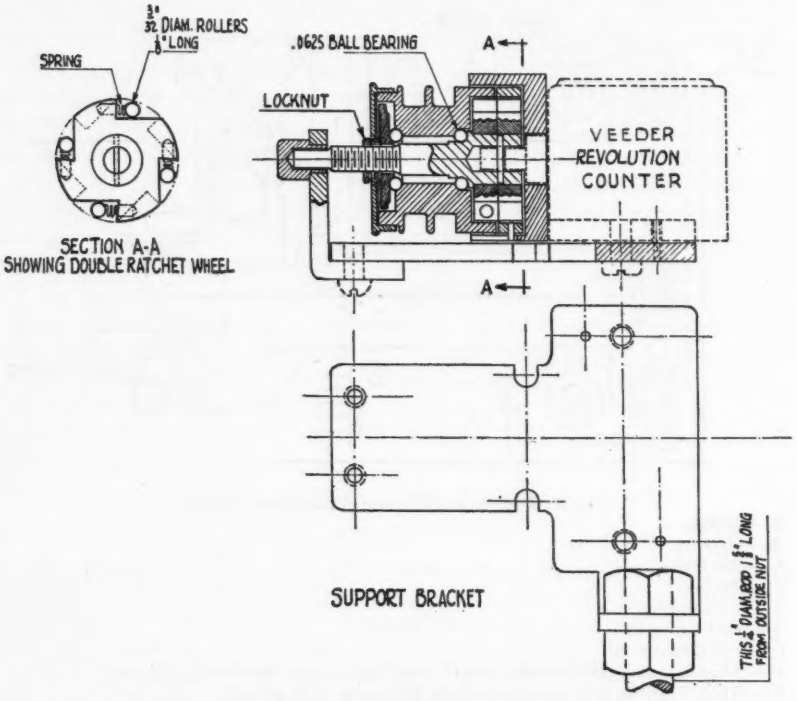

FIG. 2. Diagram of the Construction of the Work Recorder.

caused a significant change in the amounts of work performed by adrenalectomized animals.

Male rats of the Sprague-Dawley strain which weighed 180 ± 2.0 gm. were used in these experiments. These animals were free of parasites and infections. The success of the procedures described in this paper is dependent upon the use of infection-free animals. The diet was Purina dog chow.

The animal was anesthetized by the subcutaneous injection of 27.0 mgm. of phenobarbital sodium and by the intraperitoneal injection of 5.0 mgm. of cyclopal sodium. The operation was begun thirty minutes after administration of the anesthetics. The adrenal glands and the kidneys were removed by the methods described by Ingle and Griffith (1942). The pedicles of the adrenal glands and of the kidneys were ligated before these organs were

removed. The animals were then mounted on the board (fig. 3). The muscles were stimulated weakly at first and then the stimulus was gradually increased so that by the end of ten minutes it was brought to a standard intensity known to be optimal for sustaining the contraction of muscle. It is important to note that a relatively weak stimulus will induce maximal contractions during the first few minutes of stimulation but the muscle will later show

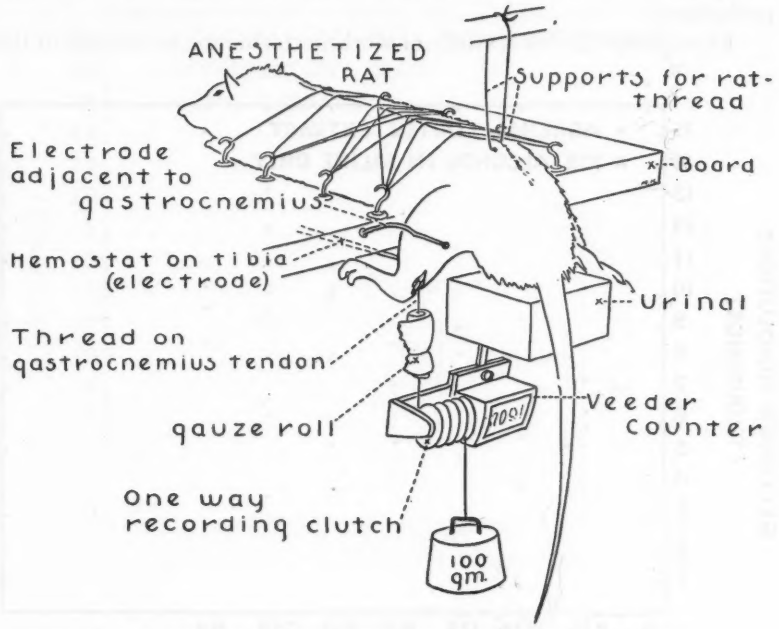

FIG. 3. Anesthetized Rat Prepared for the Stimulation of Muscle.

"fatigue" at this weak intensity whereas a strong stimulus will sustain vigorous contractions over a period of many hours.

Injections of the cortical hormones were made at the beginning of stimulation and again six hours later. Crystalline compounds were ground into a fine sesame oil suspension. The volume of oil was kept constant at 0.5 cc. per dose. Adrenal cortex extract was in a solution of 10 per cent alcohol and water and contained 0.9 per cent sodium chloride. The volume of aqueous preparations was kept constant at 1.0 cc. per dose.

Stimulation was continued until the muscle ceased to respond or for a period of twenty-four hours. The total number of recorder revolutions for the twenty-four hour period was used as an index of the efficacy of treatment.

The analyses of the amounts of glucose in the tail blood of the working rats were carried out by the method of Miller and Van Slyke (1936).

EXPERIMENTS AND RESULTS

Experiment 1 was a study of work performance as related to the dosage of cortical extract. The size of dosage was adjusted so that

each consecutive higher level represented an increase of 25 per cent. The size of dose expressed in terms of the number of grams of whole beef adrenal gland from which that dose of extract was derived and the number of animals tested at each dose are shown in relation to work performance in Figure 4. It was noted that each increase in dosage was associated with an increase in the average amount of work performed.

Experiment 2, was a study of work performance as related to the

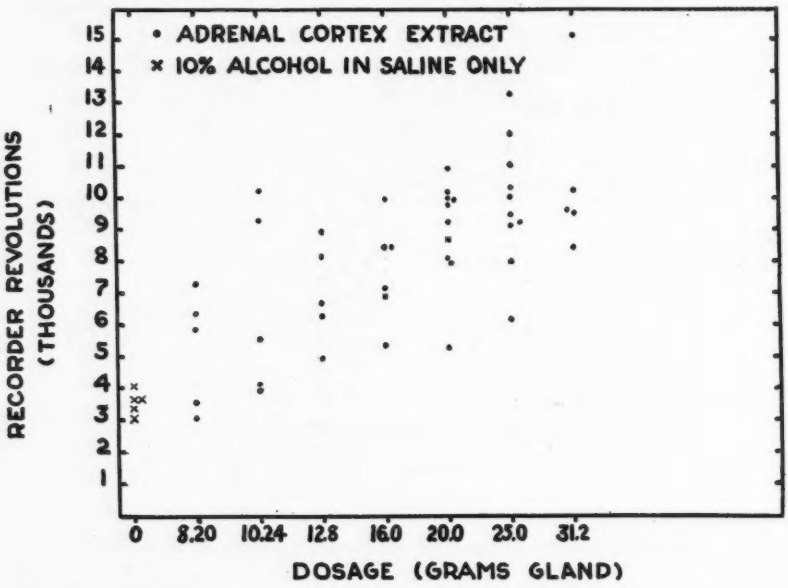

FIG. 4. Work Performance as Related to the Dosage of Adrenal Cortical Extract.

amount of 17-hydroxy-11-dehydrocorticosterone administered to the test animals. As in experiment 1, the size of dosage was adjusted so that each consecutive higher level represented an increase of 25 per cent. The size of dose and the number of animals tested at each dose is shown in relation to work performance in Figure 5. Each increase in dosage was associated with an increase in the average amount of work performed. On the basis of these results it was decided to define a unit of activity as the work equivalent of a 0.2 mgm. dose of 17-hydroxy-11-dehydrocorticosterone (administered twice during the test) and to set up a standard dose-response curve which would permit the interpolation of work performance into units of the standard.

Experiment 3 had to do with the setting up of a standard dose-response curve. Dosage levels of 0.16, 0.20 and 0.25 mg. of 17-hy-

droxy-11-dehydrocortocosterone were used. Forty-five rats were tested at each dosage level. The average value for work at each dose was plotted against the logarithm of the dose (fig. 6). The mean values for work at each of the three dosage levels, the standard deviations of the means and the standard deviations of the differences

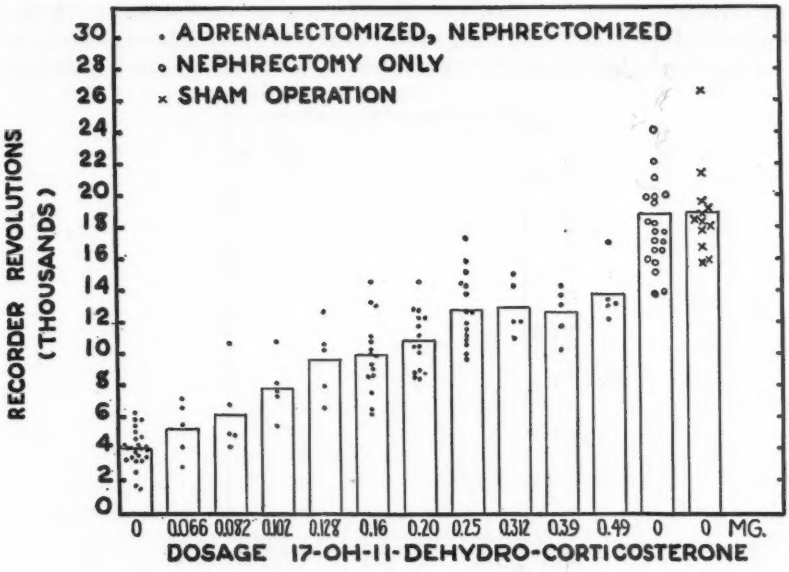

FIG. 5. Work Performance as Related to the Dose of 17-hydroxy-11-dehydrocorticosterone.

between the means are given in table 1. These differences between means are statistically reliable.

TABLE 1. AVERAGES AND VARIABILITY OF THE VALUES FOR TOTAL WORK UPON WHICH THE STANDARD CURVE (FIG. 6) IS BASED

Dose Mg.	Number Animals	Mean	Sigma Distribution	Sigma Mean	Diff. Between Means	Sigma Diff.	Diff. Sigma Diff.
0.16	45	9439	2789	415	1857	538	3.45
0.20	45	11296	2294	342			
0.25	45	12982	2560	382	1686	513	3.29

By the use of data such as is given in table 1, it is possible to calculate, from a statistical standpoint, the number of animals required for the assay of a single sample. The following formula given by Munch (1931) was used: $N = (k \times \sigma/\text{diff})$.[2] The arbitrarily selected

chance to be taken (k) was 2.58 standard deviations (σ) the odds of which are 100 to 1 against the occurrence of a deviation as great or greater. It was desired that the error (diff) should represent a change in dose of less than 25 per cent. The dosages used in the standard curve (fig. 6 table 1) represent successive increases of 25 per cent so that the differences between the mean values for work can be used to represent "diff." When the distribution of values for work by animals given a dose of 0.2 mg. 17-hydroxy-11-dehydrocorticosterone are used in calculating "N" the above formula is substituted as fol-

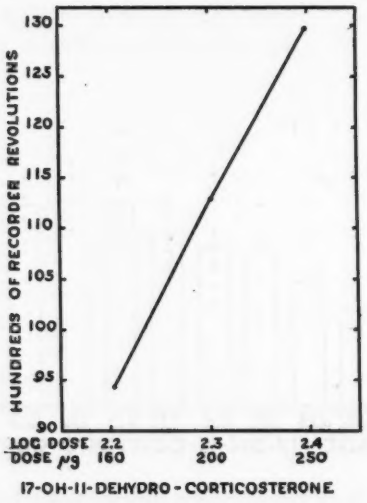

FIG. 6. Standard Dose-Response Curve Based Upon the Average Performance of 45 Rats at Each of the Three Dose Levels.

lows: $N = (2.58 \times 2294/1686)^2$ so that "N" is 12.32. When the distribution of values for work by animals given 0.16 mg. of 17-hydroxy-11-dehydrocorticosterone is used in the calculation the value for "N" is 14.97. When the distribution of values for work by animals given 0.25 mg. of 17-hydroxy-11-dehydrocorticosterone is used in the calculation the value for "N" is 15.3. On the basis of these results it was decided to use a minimum number of 15 rats for each individual assay.

A further step is taken to increase the probability that the "error of assay" will not deviate from the "true value" by more than 25 per cent. After 15 animals have been tested the average value for work and the standard deviation of the average are calculated. The ratio between the standard deviation of the average and the smallest change in number of recorder revolutions (1686) which represents a 25 per cent increase in dosage on the standard curve (fig. 6) is required to be 2.0 or greater. If this requirement is not met by the use of 15 animals

the number is increased until the requirement is satisfied. From the probability standpoint, a ratio of 2.0 means that the odds against the chance occurrence of a deviation as great as 1686 are 21 to 1.

In actual practice the ratio between the standard deviation of the average and the value 1686 is only rarely smaller than 2.0 when 15 animals are used. In thirty consecutive assays the average ratio obtained was 2.97 with a range of 2.02 to 5.65.

Experiment 4, was an empirical test of the reliability of the method of assay. The standard, 17-hydroxy-11-dehydrocorticosterone was assayed at different times of the year. This data is summarized in table 2. Acceptable checks were obtained in all of the cases. Similarily, acceptable checks have been consistently obtained in the assay of adrenal cortical extracts during the past two years in this laboratory.

TABLE 2. ASSAYS OF 17-HYDROXY-11-DEHYDROCORTICOSTERONE AT DIFFERENT TIMES OF THE YEAR

Time	Units/mg.	Time	Units/mg.
February 1942	4.75	December 1942	4.55
July 1942	5.54	February 1943	5.59
September 1942	4.57	April 1943	4.62
November 1942	4.98	Average	4.92

Experiment 5, was a study of the level of blood sugar in relationship to work performance under different conditions and treatment. The samples of blood were taken at the end of seven hours of muscle stimulation. The failure of adrenelctomized rats and of adrenalectomized-nephrectomized rats to continue work was associated with hypoglycemia whereas those animals having intact adrenal glands and the adrenalectomized-nephrectomized animals treated with 17-hydroxy-11-dehydrocorticosterone were capable of continuing work and of sustaining the level of blood sugar better than did untreated animals. The data are summarized in table 3.

TABLE 3. AVERAGE VALUES FOR WORK* AND BLOOD-SUGARS FOR MALE RATS (180 GMS.) WORKING UNDER THE CONDITIONS OF THE INGLE WORK TEST

Experimental condition	Rate of work at seven hours	Blood-sugars (Mg.%) at seven hours
Adrenalectomy & nephrectomy 0.5 mg. E. per dose	16.3	64
Adrenalectomy & nephrectomy 0.5 mg. Doc-Ac per dose	1.8	45
Adrenalectomy & nephrectomy untreated	0.9	39
Adrenalectomy only untreated	7.0	39
Nephrectomy only untreated	14.0	73
Normal sham untreated	16.0	87

Note: Each experimental group was represented on each day that an experiment was carried out. The above averages are each based on 5–7 rats.

* Number of recorder revolutions per minute.

E = 17 hydroxy-11 dehydrocorticosterone

Doc-Ac = 11-desoxycorticosterone acetate.

DISCUSSION

The objective of this study was to develop a method of assay which would be objective, sensitive, reliable and specific in response to those cortical steroids which influence the metabolism of carbohydrate and sustain the resistance of adrenalectomized animals to severe stress. The method described here meets each of these requirements. It is not a simple method. The procedures and the test conditions are exacting and are not easily mastered. Consequently, this method is likely to have only a limited usefulness. More simple methods which will meet the same purpose are desirable.

Statistical evaluations of the reliability of a quantitative method concern only the variability due to chance selection. Changes in results due to changes in experimental conditions are not estimated in such calculations. The most sound procedure to follow in biologic assays is to make simultaneous comparisons of the standard and the "unknown." Since it has not been practical to follow this procedure an attempt has been made here to omit the repeated use of the standard by attaining a rigid control over the experimental conditions. Our data indicate that by the complete control of temperature, the use of infection-free animals and by standardization of all other details of the assay procedure the results of any individual assay can be reliably interpolated from a standard curve.

The selection of the adrenalectomized-nephrectomized rat as a test animal was based upon the following considerations. In order to shorten the time required by the test it was necessary to impose an additional stress upon the adrenalectomized animal. In the procedure used earlier (Ingle, 1936; 1939) a subcutaneous injection of water was made at the beginning of work and again six hours later in order to increase the stress. This was an intermittent stress whereas it was desirable to use a steady stress. In addition, the administration of water is known to cause a lowering of the amounts of sodium and chloride ions in the blood serum. It was thought to be desirable to avoid changes in electrolyte balance in this method which was intended to be responsive to principles effecting carbohydrate and not electrolyte metabolism. Since one of the important sites of action of the cortical hormones on electrolyte balance is the kidney it was assumed, on hypothetical grounds, that it would be desirable to devise a method of assay in which the effect of renal functions would be eliminated. Finally and of greater importance, favorable results were obtained by using the adrenalectomized-nephrectomized rat. As was shown by Ingle and Kendall (1936), the adrenalectomized-nephrectomized rat is unable to work for more than ten hours when treatment is omitted but will continue to work for longer than twenty-four hours when treatment is optimal. Almost all of the untreated animals collapse during the afternoon of the same day stimulation is begun and all of the animals given optimal treatment with cortical hormones continue

to work for longer than twenty-four hours. Other experiments have been conducted with adrenalectomized rats in which heat, cold, toxins and fasting were each tried as an added source of stress. The most satisfactory results were obtained with the adrenalectomized-nephrectomized rat.

It was noted in experiments 1 and 2 (figs. 4 and 5) that the maximum average amount of work performed by adrenalectomized-nephrectomized rats treated with beef adrenal extracts or with 17-hydroxy-11-dehydrocorticosterone was less than the average performance of either sham-operated or nephrectomized rats having intact adrenal glands. This persisting deficiency, which has been noted in all of my earlier studies on the work performance of treated adrenalectomized rats, may be due to the absence of the adrenal medulla or to a failure to duplicate either qualitatively or quantitatively the secretion of the adrenal cortex by means of intermittent injections of the cortical hormones.

The results obtained by Ingle, Nilson and Kendall (1937) in studies of the adrenalectomized-nephrectomized rat, the results of this study (Table 2) and unpublished data on liver and muscle glycogen show that the loss of muscular responsiveness is associated with a depletion of the carbohydrate stores. These results and the additional finding that this assay method is specific for those cortical steroids which influence the metabolism of carbohydrate (Ingle, 1940a, 1940b) might be explained by the hypothesis that failure to work is due to depletion of the stores of carbohydrate. The studies of Ingle and Lukens (1941) show that depletion of carbohydrate is an important factor in the "fatigue" of adrenalectomized rats but they also observed, as did Hales, Haselrud and Ingle (1935), that the inability to work is associated with a circulatory collapse. The relationship of the circulatory failure to the failure in carbohydrate metabolism is obscure. Ingle (1942) has reviewed the evidence that the physiologic effects of the cortical hormones cannot be fully explained in terms of the known effects upon carbohydrate and electrolyte metabolism.

There is no satisfactory evidence that any of the cortical hormones will increase the ability to work of animals or patients having functionally normal adrenal glands. Although the asthenia resulting from cortical insufficiency is very marked and the effect of the C_{11} oxygenated steroids and cortical extracts in restoring the ability to work to nearly normal levels is very striking and specific it should be noted that a similar "fatigability" characterizes animals having other deficiency states or animals severely damaged by unrelated causes.

The results of these experiments support the conclusion that the muscle-work test described here is a sensitive and reliable method for the detection and quantitative estimation of the biologic activity characteristic of the C_{11} oxygenated cortical steroids.

SUMMARY

A method is described for the quantitative assay of adrenal cortical hormones by the muscle-work test using the adrenalectomized-nephrectomized rat. A unit of activity is defined as the work equivalent of a 0.2 mgm. dose of 17-hydroxy-11-dehydrocorticosterone administered twice during the test. A standard dosage-response curve was set up using 17-hydroxy-11-dehydrocorticosterone which permits the interpolation of work performance into units of the standard. The method is specific for the detection and estimation of biologic activity characteristic of the C_{11} oxygenated cortical steroids. Data illustrating the sensitivity and reliability of the test are presented.

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THE METABOLISM OF ESTRONE IN NORMAL AND PARTIALLY HEPATECTOMIZED RATS[1]

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PREVIOUS REPORTS on the urinary recovery of estrogen administered to the rat are contradictory. Zondek (1934) obtained in 72 hours only 0.6% of the activity of estrone administered orally or parenterally to adult female rats in doses of 2000 and 5000 I.U. After injecting similar quantities of estrone Dingemanse and Laqueur (1937) obtained urinary recoveries up to 2% with slightly larger amounts in the feces. Dorfman (1937), on the other hand, has reported estrogenic activity in the urine of injected immature rats equal to 10% of the injected estrone. Evidence that rat livers, when perfused *in vitro* with estrogen, are quite potent in effecting estrogen conversion (1943) led us to re-examine the question of the urinary excretion of exogenous estrogen in normal animals and in animals lacking considerable amounts of liver tissue.

MATERIAL AND METHODS

In order to obviate any possible effect of endogenous ovarian activity we employed adult male rats (of the Sherman strain). These animals were placed in the urine collecting cage in groups of 7 to 10 (see Table 1). Previous to estrone administration control urine collections were made for 72 hours. After the subcutaneous injection of 50 micrograms of estrone-in-oil (1 cc.) per rat the urines collected in the first 48 hours were kept separate from those collected in the succeeding 48 hours.

The urine specimens were brought to pH 1–2 with concentrated HCl, and autoclaved for 20 minutes at 14 lbs. pressure. Subsequent ether extraction and partition of the phenolic fractions into weak phenolic non-ketonic, weak phenolic ketonic, and strong phenolic fractions was accomplished by methods previously described (1942, 1943). The three fractions obtained were assayed by injection into spayed rats (1941), the potency of each fraction being determined from standard assay curves for α-estradiol, estrone and estriol.

In hepatectomy we followed the methods of Selye (1941). After light ether anesthesia a transabdominal incision was made just below the ribs.

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The ligatured median and left hepatic lobes were removed *in toto*, thus effecting the extirpation of 70–75% of the total liver tissue. The abdominal incision was closed with silk stitches and collodion applied. No animal was kept under anesthesia for more than five minutes and in successful operations hemorrhage did not occur. Care was taken to avoid injury to the diaphragm. Operative and post-operative mortality was 20 to 25%. The only special post-operative care employed was the intraperitoneal injection of 10 cc. of an isotonic glucose solution. Survival of the critical 24-hour period after operation led to the resumption of fairly normal activity which continued for several weeks.

RESULTS

In Table 1 are presented the data on urinary estrogenic activity in the control and post-injection series of normal and hepatectomized rats. These data demonstrate: (a) that the uninjected normal male

TABLE 1. THE URINARY ESTROGEN OF NORMAL AND HEPATECTOMIZED MALE RATS BEFORE AND AFTER THE INJECTION OF 50 MICROGRAMS OF ESTRONE PER RAT

Type of Animal	No. of Animals in Group	Injected or Uninjected	Period of Urine Collection	Urinary estrogen in micrograms per rat per forty-eight hours		
				Non-ketonic Weak Phenolic (as α-Estradiol)	Ketonic Weak Phenolic (as Estrone)	Strong Phenolic (as Estriol)
Normal	10	Uninjected	72 hrs.	0.19	0.58	<0.03
Hepatectomized	8	Uninjected	72 hrs.	0.37	0.62	<0.04
Normal	10	Injected	1st 48 hrs. after injection	1.17	2.40	4.00
Hepatectomized	7	Injected	1st 48 hrs. after injection	4.03	11.40	7.94
Normal	10	Injected	2nd 48 hrs. after injection	0.50	1.54	1.29
Hepatectomized	7	Injected	2nd 48 hrs. after injection	1.77	5.10	3.47

rats excreted in 72 hours detectable amounts of estrogenic material obtainable in the weak phenolic fractions, but none in the strong phenolic fraction, (b) that the uninjected hepatectomized rats excreted nearly twice the amount of non-ketonic weak phenolic estrogen demonstrable in the urines of non-hepatectomized males but insignificantly more weak phenolic ketonic material and no detectable strong phenolic estrogen, (c) that the activity of all three urinary phenolic fractions increased notably after estrone injection, (d) that during the first 48 hours after injection into non-hepatectomized males the non-ketonic weak phenolic activity increased some six times, the ketonic weak phenols increased approximately four times, and the strong phenolic fraction contained nearly 60% of the total estrogen,

(e) that during the second 48 hours after injection the total estrogen excreted decreased but measurably more than the control amount was obtained in each fraction, (f) that during the first 48 hours after injection there occurred in the hepatectomized males a twelve-fold increase in excretion of weak-phenolic non-ketonic estrogen, and eighteen-fold increase in weak phenolic ketone, and an excretion of strong phenolic estrogen equal to approximately 30% of the total measured estrogen, (g) that during the second 48 hours after estrone injection the hepatectomized males exhibited a decreased excretion but the amounts obtained in the weak phenolic non-ketonic and weak phenolic ketonic were respectively ± 6 times and ± 9 times the control values, and the strong phenolic estrogen again accounted for approximately 30% of the measured estrogen.

In Table 2 we present the calculated recoveries of the injected estrone assuming that the weak phenolic non-ketonic estrogen is

TABLE 2. THE CALCULATED RECOVERIES OF INJECTED ESTRONE AS THE VARIOUS URINARY METABOLITES (SEE TEXT)

Type of Animal	Collection Period	Estrogen recovered per rat							
		Estradiol		Estrone		Estriol		Totals	
		μgm.	%	μgm.	%	μgm.	%	μgm.	%
Normal	1st 48 hrs. after injection	0.97	1.9	1.82	3.6	4.00	8.0	6.79	13.6
Hepatectomized	1st 48 hrs. after injection	3.66	7.3	10.79	21.6	7.94	15.9	22.39	44.8
Normal	2d 48 hrs. after injection	0.30	0.6	0.96	1.9	1.29	2.6	2.55	5.1
Hepatectomized	2d 48 hrs. after injection	1.77	3.5	5.10	10.2	3.47	6.9	10.34	20.7

α-estradiol, the ketonic weak phenol is estrone, and the strong phenol is estriol. On this basis there is recovered from the non-hepatectomized males 18.7% of the injected estrone in 96 hours, whereas 65.5% of the material appears in the urine of hepatectomized males over the same period. It is notable that the proportion of non-ketonic weak phenol is practically constant for each collection period from either type of animal, but though the proportion of "estrone" and "estriol" varies little in the two collection periods within groups there is considerable alteration in their ratio in the hepatectomized group. During the first forty-eight hours after injection the ratio of recovered "estrone" to "estriol" is approximately 1:2.2 in the normal males whereas it is about 1:0.8 in the hepatectomized males; during the second 48 hours the corresponding ratios are 1:1.4 and 1:0.6.

A repetition was undertaken in a second group of 10 normal male rats; each was injected with 50 micrograms of estrone-in-oil. The urine collected for 96 hours was divided into two equal aliquots. The first

was hydrolyzed in the usual manner; the second was not hydrolyzed but extracted directly with ethyl ether. The total phenolic fractions of each of these extracts were assayed directly. The assay result was identical for the two aliquots, 82.5 R.U. (estrone equivalent) per aliquot. These data suggest that all of the estrogen excreted into the urine is in the "free" state, since hydrolysis failed to increase the output. The total activity for the 96-hour period, 165 R.U. is somewhat less than the sum of the activities of the various fractions in the previous experiment which was 215 R.U.

DISCUSSION

What do these data signify? If we assume that the estrogenic activity of the three principal urine fractions are accountable to α-estradiol, estrone, and estriol respectively, then certain definite consequences follow. These are: (a) that injected estrone is converted in normal male rats principally to estriol and in part to estradiol, (b) that over 80% of the injected estrone cannot be accounted for in such animals on the basis of urinary recoveries, (c) that in partially hepatectomized male rats injected estrone is also converted to estradiol and estriol but that a much larger proportion is recovered unchanged, (d) that the conversion of estrone to estriol is differentially reduced by the hepatectomy whereas the conversion to estradiol is scarcely affected, (e) that all estrogens are excreted in increased amounts after partial hepatectomy so that only 35% of the injected estrone remains unaccounted for in terms of urinary recovery. We may therefore ascribe to the liver two major roles in the metabolism of estrone: one is that it acts markedly to convert estrone to estriol; the second is that it effects the conversion of all the estrogens to material that does not appear in the urine as active phenolic estrogen. The latter inactivation has been generally accepted since the work of Zondek (1934, 1941) on the disappearance of estrogenic activity after the incubation of estrogen with liver brei, the more recent work of Heller (1940; 1943) and Twombly and Taylor (1942) on similar inactivation by liver slices, the studies demonstrating that liver damage increases the effect of endogenous (Talbot, 1939) and exogenous (Pincus & Martin, 1940) estrogen whereas exogenous estrogen implanted in sites drained by the hepatic portal system exerts little or no effect (Biskind 1941, 1939), (Israel *et al.*, 1937) and the report that estrone is inactivated rapidly on perfusion through dog's liver (Israel *et al.*, 1937). The role of the liver as an organ effecting the conversion of estrone to estriol has not, however, been recognized although our recently reported experiments (Schiller and Pincus, 1943) on the perfusion of rat livers indicate that "estriol" is a major conversion product of α-estradiol. We have unpublished data on estrone perfused through rat livers indicating its conversion to estriol also. Heller (1940) has presented evidence that rat liver slices convert estrone to α-estradiol, but that

this is a property of other tissues as well. The data of this paper showing a practically unaltered ratio of estradiol excretion after estrone injection into the hepatectomized rats indicate that in the over-all bodily activities effecting this conversion the role of liver tissue is a minor one, though activity of the liver in this conversion probably occurs to some extent.

Another indication of these data worth noting is the probability that estradiol is the primary estrogen produced in male rats. We deduce this from the fact that partial hepatectomy is followed by increased activity attributable to "estradiol" but scarcely any increase attributable to "estrone." We have shown (Schiller and Pincus, 1943) that α-estradiol is converted to estrone by the perfused rat liver. In these experiments we may deduce that such conversion is largely inhibited by the hepatectomy thus causing an increased excretion of estradiol. Otherwise we would have to infer that estrone is more rapidly destroyed on hepatectomy and our data on excretion of injected estrone directly contradict this.

The data of our second experiment with intact male rats give no indication of detectable conjugation of post-injection urinary estrogen. This is somewhat surprising in view of the finding that urinary estrogen in human and equine urine is excreted as esterified material. Dingemanse and Laqueur (1937) also were unable to obtain evidence for the esterification of injected estrogen in the rat, and our data (1943) on rat liver perfusion gave no indication of esterification by the liver. Heller (1940) also mentions that hydrolysis of liver-slice estrogen preparations fails to give increased activity. Since the data on the hepatectomized animals definitely implicate the liver as a prime agent in inactivation and since it seems that this inactivation cannot be attributed to detoxification, another avenue for the disdisposition of active estrogen must be sought. Alternatively we must consider that the foregoing attempts to free esterified estrogen by various hydrolyses are not adequate. While the methods of Dingemanse and Laqueur (1937) were not optimal by modern standards, our method has yielded the largest recoveries yet reported in the literature.

Nor can we on the basis of available evidence attribute the missing estrogen to excretion in the feces. Dingemanse and Laqueur (1937) were unable to account for more than 3% of injected estrogen in the feces.[2] Zondek (1934) and Dingemanse and Laqueur (1937) demonstrated that the 3-benzoate of estrone is not destroyed as rapidly as the free hormone. This may be due to protection of the phenolic ring, but Dingemanse and Laqueur (1937) attribute slow inactivation to slow absorption. Furthermore Pincus and Graubard

[2] In a preliminary experiment we obtained 100 R.U. of estrogen in normal male rat feces in 96 hours after estrone injection compared with an average urinary excretion of 190 R. U.

(1942) were unable to obtain phenolases from animal livers. The experiments of Cantarow *et al.* (1942) with the dog offer the possibility of estrogen excretion and storage in the bile. The experiments of Zondek (1934) and Dingemanse and Laqueur (1937) on the extraction of the entire animal give no support for this possibility since the bodies yielded only minor amounts by six to twelve hours after injection. In our partially hepatectomized animals the bile ducts always were left intact, though functional liver tissue was of course markedly reduced. Recently Cantarow *et al.* (1943) have advanced the hypothesis that the liver stores injected estrogen in an inactive form and then excretes it in "reactivated" (and presumably in the original) form into the bile. Since in their experiments the interconversion of estrogens has not been examined, the factual basis for their hypothesis is clearly incomplete.

The foregoing evidence, though admittedly incomplete, is consistent with the conclusion that estrogen degradation *in vitro* probably occurs through chemical attack on the fourth ring and suggests that Marrian's (1938–39) hypothesis of the formation of a 16–17 diketone from estriol or some similar process may be involved. The recent data of Segaloff (1943) on the protection of 17-0H estrogen esters from hepatic destruction confirm the notion of attack on ring D. In the rabbit the formation of β-estradiol may account for a considerable degree of inactivation, but this certainly does not apply to the human (Pearlman, 1943; Pincus, 1942), nor do our data support this possibility in the rat. If β-estradiol were the non-ketonic weak estrogen of the rat urines of this experiment then the amounts calculated for this fraction should be increased 100 times since by our assay method β-estradiol has 1/100th the activity of α-estradiol; this leads to impossibly large recoveries in both series of animals. Whatever the final inactivation products may be our data indicate the preponderant role played by the liver in effecting their production.

It should be noted that these experiments were conducted during the three week period (Higgins and Anderson 1931, Brues et al 1936) when liver regeneration following hepatectomy is completed. In spite of the fact that often in a few days almost complete tissue restoration occurs our data imply that functional restoration may be less rapid. This is in accord with Higgins and Anderson (1931).

SUMMARY

The phenolic fractions of the pooled urines of groups of 7 to 10 male rats were divided into non-ketonic weak phenols (estradiol), ketonic weak phenols (estrone) and strong phenols (estriol). These three fractions were assayed for estrogenic activity in urine collected before and after partial hepatectomy and before and after the injection of 50 micrograms of estrone per rat.

The estrogen assay data indicate that normal male rats effect

the conversion of injected estrone to non-ketonic weak phenolic and strong phenolic estrogen. On the assumption that the activity of the three phenolic fractions is accountable to α-estradiol, estrone and estriol, 18.7% of the estrone injected into normal male rats can be accounted for in the urinary excretion for 4 days following injection. The urinary estrogen excreted by the normal male rats after estrone injection appears to be free, not esterified.

The endogenous estrogen excreted into the urine following partial hepatectomy increases in amount particularly in the non-ketonic weak phenolic fraction. After estrone injection into hepatectomized male rats the urinary estrogen titers increase markedly. It may be calculated that 65.5% is recovered in terms of urinary metabolites.

While hepatectomy leads to increased estrogenic activity in all phenolic urine fractions after estrone injection, the strong phenolic estrogen increases a little over two times, the weak-phenolic non-ketonic approximately four times and the weak phenolic ketonic nearly six times in a 96-hour collection period. This is taken to indicate that the conversion of estrone to estriol is most markedly interfered with by liver lobe extirpation.

We are much indebted to Prof. L. C. Wyman for discussion and criticism.

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NOTES AND COMMENTS

NO EFFECT ON ADRENAL HYPERTROPHY OF SUBDIAPHRAGMATIC VAGOTOMY UNDER CONDITIONS OF DECREASED BAROMETRIC PRESSURE[1]

IT IS KNOWN that the adrenal glands hypertrophy when animals are subjected to decreased barometric pressure (Armstrong and Heim, 1938; Dohan, 1942; Langley and Clarke, 1942; Thorn, Jones, Lewis, Mitchell and Koepf, 1942). It has been suggested that the stimulus causing the adrenal hypertrophy under these conditions is an initial increased utilization of carbohydrate and a rapid depletion of the carbohydrate stores of the body (Lewis, Thorn, Koepf and Dorrance, 1942). Furthermore, injections of insulin also cause adrenal hypertrophy (Schenk and Langecker, 1935; Randall, 1940; Mikeleitis, 1940; Miller and Riddle, 1941). The fact that vagotomy abolishes the fall in blood sugar in adrenalectomized rats under conditions of anoxia and that the insulin content of the blood of adrenalectomized rats is increased on exposure to low oxygen pressures has been interpreted to indicate that the vago-insulin system is activated by hypoxia (Feldman, Cortell and Gellhorn, 1940). Therefore, the following hypothesis was set up: under conditions of low oxygen pressure there is an increased secretion of insulin brought about by excitation of the vago-insulin system; the increased insulin content of the blood *per se* or the accompanying decreased blood sugar then stimulate the adrenal cortex either directly or through the pituitary. This hypothesis was tested by studying the effect of vagotomy on adrenal hypertrophy under conditions of decreased oxygen pressure.

Thirty-six male rats weighing 200 to 250 gm. were divided into 4 groups of 9 animals each. The first was used as a control group; the second was not allowed to eat or drink for 48 hours; the third was subjected to a barometric pressure of 294 mm. Hg (24,000 feet) for 48 hours, during which time the animals did not eat or drink; and the fourth group underwent bilateral subdiaphragmatic vagotomy and one week later was subjected to the same reduced pressure as group three. The animals of each group were then killed and the adrenals removed and weighed.

The results given in Table 1 show primarily that vagotomy failed to prevent the hypertrophy of the adrenal gland occurring under conditions of decreased barometric pressure. The normal group subjected to lowered barometric pressure showed a hypertrophy of 36 per cent, the average

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[1] Aided by a grant from the Josiah Macy Foundation, administered by Professor E. Gellhorn.

TABLE 1. ADRENAL WEIGHT OF RATS AFTER 48 HOURS OF EXPOSURE TO A BAROMETRIC PRESSURE OF 294 MM. HG.

	Control Group 1	Group 2 No Food or Water for 48 Hr.	Group 3 294 mm. Hg 48 Hr.	Group 4 294 mm. Hg 48 Hr. Vagotomized
Average	*mg.* 24.3±1.28*	*mg.* 25.3±0.87	*mg.* 34.3±1.08	*mg.* 35.66±1.69

* Standard Error.

adrenal weight for this group being 34.3 mg. per rat while for the control group it was 24.3 mg. The average adrenal weight for the vagotomized group subjected to reduced barometric pressure was 35.66 mg. per rat, which is not essentially different from the group of normal rats also subjected to reduced pressure.

Summary. Sub-diaphragmatic vagotomy had no effect on the hypertrophic response of the adrenal gland to anoxia when the animals were subjected to a barometric pressure of 294 mm. Hg for 48 hours.

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ASSOCIATION NOTICE

ANNOUNCEMENT OF THE ANNUAL MEETING

The Twenty-Seventh Annual Meeting of The Association for the Study of Internal Secretions will be held in Chicago, Illinois, on Monday, June 12, 1944, and if sufficient papers of merit are available the Meeting will be extended to two days, June 12 and 13. The Stevens Hotel will be the headquarters for registration and for the scientific and business sessions. Room reservations should be made directly with the Stevens Hotel. Hotel and transportation reservations should be made immediately.

The Chairman of the Local Committee is Dr. Willard O. Thompson, 700 North Michigan Avenue, Chicago.

PRESENTATION OF PAPERS

1. The title of the paper and four copies of a comprehensive abstract should reach the President, Dr. E. Kost Shelton, 921 Westwood Boulevard, Los Angeles, California, not later than March 15, 1944.

Abstracts submitted should be in proper form for printing in the program. Not more than the first 200 words can be included in the printed abstract.

2. Non-members who wish to present papers must have their titles and abstracts introduced by members.

3. The abstracts of papers will be considered by the Program Committee and the final program announced about May 10, 1944.

4. Papers will be limited to ten minutes for presentation. A ten-minute presentation is designed for condensed discussion of new investigations, either in laboratory or clinical phases of endocrinology. Previous publication or presentation before other societies of national membership may be cause for omitting a paper from the program.

5. Papers presented at the Annual Meeting may be submitted for publication to the Editor of Endocrinology or The Journal of Clinical Endocrinology. Such submission is invited. Acceptance of a paper for the program does not necessarily mean its acceptance for publication.

You are invited to bring this announcement to the attention of your colleagues and associates who have endocrine research in progress.

NOMINATION OF OFFICERS

The By-Laws of the Association provide that nominations for all elective offices shall be made by a Nominating Committee and forwarded to the Secretary at least sixty days before the annual meeting. The Nominating Committee for the current year, appointed by the President and accepted by the Council, is as follows:

Dr. E. Perry McCullagh (Chairman), Cleveland Clinic, Cleveland, Ohio.
Dr. C. N. H. Long, Yale University School of Medicine, New Haven, Connecticut.
Dr. Warren O. Nelson, Department of Anatomy, Wayne University, Detroit, Michigan.

The By-Laws also provide (Article V, Section 2) that "Any member of the Association may submit nominations to the Nominating Committee for its consideration." Nominations may be sent to the Chairman, Dr. E. Perry McCullagh, before March 15, 1944.

The terms of the following officers expire at the time of the Annual Meeting in Chicago in 1944:

President-Elect	Carl R. Moore
Vice-President	John C. Burch
Secretary-Treasurer	Henry H. Turner

Council Members

P. E. Smith M. A. Goldzieher George W. Thorn Edgar Allen (deceased) (Term expires 1946)

Publication Board

E. C. Hamblen Carl R. Moore

THE E. R. SQUIBB & SONS AWARD

The E. R. Squibb & Sons Award of $1000.00 was established in 1939, and was given first in 1940 to Dr. George W. Corner, in 1941 to Dr. Philip E. Smith, and in 1942 to Dr. Fred C. Koch. A special Committee of five members of the Association chooses an investigator or investigators in the United States or Canada for one of the best contributions to endocrinology.

THE CIBA AWARD

The Ciba Award to recognize the meritorious accomplishment of an investigator not more than 35 years of age in the field of endocrinology was established in 1942, but no recipient was selected due to lack of time. The work cited may be either in the field of preclinical or clinical endocrinology. The Award is for $1200.00. If the recipient should choose to use the Award toward further study in a

laboratory other than that in which he is at present working, the Award will be increased to $1800.00. The option is left entirely to the recipient.

Each member has the privilege of making one nomination for each Award. A nomination should be accompanied by a statement of the importance of the nominee's contributions in endocrinology and by a bibliography of the nominee's most important publications, and reprints if possible. Five copies should be sent to the Secretary, Dr. Henry H. Turner, 1200 North Walker Street, Oklahoma City, Oklahoma, not later than March 15, 1944.

E. KOST SHELTON, *President*
HENRY H. TURNER, *Secretary*